Remarks

Claims 18-107 are pending in the subject application. Applicant acknowledges that claims 64-107 have been withdrawn from further consideration as being drawn to a non-elected invention. By this Amendment, Applicant has amended claims 18, 31, and 64 and canceled claims 98-107. Support for the amendments can be found throughout the subject specification and in the claims as originally filed. Entry and consideration of the amendments presented herein is respectfully requested. Accordingly, claims 18-63 are currently before the Examiner. Favorable consideration of the pending claims is respectfully requested.

Applicant notes that withdrawn claims 64-97 are directed to methods of use of the claimed compositions. Applicant respectfully requests that withdrawn claims 64-97 be rejoined in the subject application upon an indication of an allowable composition of matter claim from the elected invention in accordance with MPEP §821.04. Applicant will amend the method claims to recite each limitation of the allowed composition claim upon rejoinder in the application.

Claim 18 is objected to for reciting the acronym "FIV." Applicant has amended claim 18 to recite "feline immunodeficiency virus" with the acronym "FIV" in parentheses, in accordance with the Examiner's helpful suggestions. Accordingly, reconsideration and withdrawal of the objection is respectfully requested

Claims 31-63 are rejected under 35 USC §112, first paragraph, as nonenabled by the subject specification. The Examiner acknowledges that the specification is enabled for a vaccine for domestic cats comprising FIV inactivated whole virus or inactivated FIV-infected cells comprising subtypes A and D, but asserts that the specification does not enable a vaccine comprising all FIV-subtype immunogens and for use in all cats, including wild cats. In addition, the Examiner cites the Yamamoto *et al.* (2002) reference as evidence to support the rejection.

Applicant respectfully asserts that claims 31-63 are enabled by the subject specification. Although at page 3, lines 4-5, of the Office Action the Examiner acknowledges that the immunogens of the composition are from "at least two different FIV subtypes," Applicant also notes that under this rejection the Examiner indicates at page 4, lines 2-3, of the Action that the claims encompass a "single immunogen subtype." Applicant respectfully submits that claim 31 specifically recites that the immunogen(s) of the vaccine composition are from or comprise "at least two different FIV

subtypes." Thus, the claims encompass compositions wherein immunogens of two or more FIV subtypes are provided.

The Examiner also asserts that the subject specification does not enable the use of all immunogen subtypes other than a combination of FIV subtype A and D immunogens. Applicant respectfully asserts that the subject specification enables the use of any immunogen comprising at least two different subtypes. The Examiner has not provided any basis to support the assertion that the subject specification is not enabled for compositions comprising "at least two different FIV subtypes." Under the authority of *In re Marzocchi*, 169 USPQ 367 (CCPA 1971), Applicant's statements must be taken as true unless the Patent Office can recite specific reasons to doubt the validity of those statements. The vaccine compositions disclosed in Table 2 of the Yamamoto *et al.* (2002) reference are directed to single subtype compositions and do not provide any teachings regarding the effectiveness of Applicant's claimed invention which utilizes immunogens of more than one subtype. Applicant respectfully maintains that an ordinarily skilled artisan can readily make and use the claimed invention.

Also under this rejection, the Examiner appears to assert that the subject specification only enables the claimed composition wherein the animal susceptible to FIV infection is a domestic cat. Applicant respectfully asserts that the claimed composition can be used to induce an immune response against FIV in any animal susceptible to infection by FIV and is enabled for use in both wild and domestic cats. Attached with this Amendment is a publication by Nishimura *et al.* (1999) which shows that wild cats can be infected with an FIV strain that infects domestic cats. Also attached with this Amendment is a publication by Briggs and Ott (1986) which shows that wild cats can be infected with feline leukemia virus (FeLV) of domestic cats and a publication by Citino (abstract 1988) showing that vaccines against domestic FeLV strains have been used in immunizing wild cats against FeLV. In addition, although FIV that infects wild cats may not be identical in genomic sequence to FIV strains that infect domestic cats, a significant amount of homology exists between them. The publication by Brown *et al.* (1994), a copy of which is attached with this Amendment, shows that lions in Africa infected with FIV have antibodies that cross-react with antigens from a strain (Petaluma) of FIV that infects domestic cats (see Figure 1 of Brown *et al.*). Given that domestic cat strains of FIV can infect wild cats, and that strains of FIV that infect wild

cats induce antibodies that cross-react with strains of FIV that infect domestic cats, and given that FeLV infects wild cats and FeLV vaccines are being used to immunize wild cats, the ordinarily skilled artisan would reasonably expect that the multi-subtype vaccines of the present invention would also be efficacious in wild cats, both against domestic cat FIV strains and non-domestic FIV strains infecting wild cats.

The Examiner also appears to assert under this rejection that the subject specification does not enable the use of recombinant viral vector FIV constructs as an immunogen. Applicant notes however, that at page 3, lines 20-22, of the outstanding Office Action that the Examiner also states that it "would require undue experimentation to provide a vaccine comprising any immunogens other than those disclosed in Yamamoto *et al.*, or a viral vector vaccine" (emphasis added). This statement seems to suggest that viral vector vaccines are considered enabled by the Examiner. Regardless, Applicant respectfully asserts that viral vector constructs are enabled for use in the present invention. Attached with this Amendment is a publication by Tellier *et al.* (1998) which shows that cats receiving a canarypox-FIV vector construct as an immunogen exhibited an immune response to FIV (see Tables 1 and 2 of Tellier *et al.* (1998)). In addition, as noted previously in these remarks, the single subtype vaccine compositions disclosed in Table 2 of the Yamamoto *et al.* (2002) reference do not provide any teachings regarding the effectiveness of Applicant's claimed invention. Thus, Applicant respectfully asserts that an ordinarily skilled artisan would expect that recombinant viral vector FIV constructs can be used in the present invention.

In view of the above remarks, reconsideration and withdrawal of the rejection under 35 USC §112, first paragraph, is respectfully requested.

Claims 18-30 and 44-49 are rejected under 35 USC §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. Applicant respectfully asserts that there is adequate written description in the subject specification to convey to the ordinarily skilled artisan that they had possession of the claimed invention. The Examiner asserts that the claimed cells are derivatives of deposited cells that have not been clearly identified by their "identifying characteristics" and, therefore, a deposit of the claimed cells is required. Applicant respectfully asserts that she is the first to teach a feline T cell

that is IL-2 independent in the absence of infection by FIV. Applicant has also deposited a species (the cell line designated as FeT-J; ATCC accession number CRL 11967) of the claimed cell. U.S. patent law does not require that an Applicant for patent describe every species within a claimed genus in order to satisfy the written description requirement of 35 USC §112. The deposit of the FeT-J cell line was made under the terms of the Budapest Treaty, as is indicated at page 7, line 23 through to page 8, line 4, of the subject specification. Moreover, Applicant respectfully asserts that an ordinarily skilled artisan can obtain the cells deposited with ATCC under accession numbers CRL 11967, 11968, 11975, 11976, 10772, and 10775 and can readily determine the "identifying characteristics" thereof. Identifying characteristics include, for example, the particular cell surface antigens expressed by the cells. A cell surface antigen expression profile can be readily determined using standard methods and materials known in the art. Accordingly, reconsideration and withdrawal of the rejection under 35 USC §112, first paragraph, is respectfully requested.

Claims 18-63 are rejected under 35 USC §112, second paragraph, as indefinite. Applicant respectfully asserts that the claims as filed are definite. Specifically, claims 18-63 are rejected because they recite "feline-derived," "derived," or "derived from." The Examiner indicates that it is unclear what components are retained in the final product when they are derived from another product. Applicant respectfully asserts that the term "derived" does not render the claims indefinite or unclear. In the context of the claims directed to cells, the term "feline-derived" indicates that the cells are feline cells. However, by this Amendment, Applicant has amended claims 31 and 64 to delete the term "derived." Claims 44-49 are rejected because "identifying characteristics" is not defined. The ordinarily skilled artisan would understand that identifying characteristics of a cell are those characteristics that can be used to distinguish the cell from other types of cells or cell lines. Claims 32-34, 38-49, 51-54, and 59-62 are rejected on the grounds that the phrase "partial FIV" is unclear. The Examiner asserts it is unclear as to what structural components are required for a partial HIV. Applicant respectfully asserts that reference to a partial FIV virus would be understood by an ordinarily skilled artisan to mean an FIV virus that is not complete, *i.e.*, a virus that is missing one or more viral components. Accordingly, reconsideration and withdrawal of the rejection under 35 USC §112, second paragraph, is respectfully requested.

Claims 18-23, 27, and 27 are rejected under 35 USC §102(b) as anticipated by Yamamoto *et al.* (1991). In addition, claim 24 is rejected under 35 USC §103(a) as obvious over Yamamoto *et al.* (1991) and further in view of Francis (WO 94/06471). The Examiner indicates that the Yamamoto *et al.* reference discloses the development of two IL-2 independent feline lymphoid cell lines chronically infected with FIV and that the Francis reference discloses the envelope sequence of the Petaluma strain of FIV in Figure 2, which contains SEQ ID NO: 1 of the subject application. Applicant respectfully asserts that the cell lines disclosed in Yamamoto *et al.* (1991) require FIV infection for IL-2 independence, whereas the claimed cell lines do not require FIV infection for IL-2 independence. Thus, the cited references do not teach or suggest an IL-2 independent feline T cell line that does not require FIV infection for that IL-2 independence. Accordingly, reconsideration and withdrawal of the rejections under 35 USC §§102(b) and 103(a) is respectfully requested.

Claims 18-63 are rejected under the doctrine of "obviousness-type" double patenting over claim 1-16 of U.S. Patent No. 6,544,528. In addition, claims 18-30 are rejected under the doctrine of "obviousness-type" double patenting" over claims 1-38 of U.S. Patent No. 6,605,282. Claims 27 and 28 are provisionally rejected under the judicially created doctrine of "obviousness-type" double patenting over claims 19-63 of co-pending Application No. 10/408,701. Applicant respectfully submits that the claims in the subject application are not obvious over claims 1-16 in the '528 patent, claims 1-38 in the '282 patent, or claims 19-63 in the '701 application. However, in order to expedite prosecution of the subject application, Applicant has submitted a Terminal Disclaimer with this Amendment which obviates these rejections over the cited patents. Accordingly, reconsideration and withdrawal of the "obviousness-type" double patenting rejections is respectfully requested.

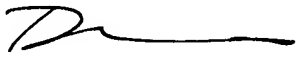
It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicant's agreement with or acquiescence in the Examiner's position.

In view of the foregoing remarks and amendments to the claims, Applicant believes that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Applicant invites the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Copy of Nishimura *et al.* (1999)
Copy of Briggs and Ott (1986)
Copy of Citino abstract (1988)
Copy of Brown *et al.* (1994)
Copy of Tellier *et al.* (1998)

JOURNAL OF VIROLOGY, Sept. 1999, p. 7916–7921 Vol. 73, No. 9
0022-538X/99/$04.00+0

Interspecies Transmission of Feline Immunodeficiency Virus from the Domestic Cat to the Tsushima Cat (*Felis bengalensis euptilura*) in the Wild

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Received 11 February 1999/Accepted 15 June 1999

Feline immunodeficiency virus (FIV) was isolated from a wild-caught Tsushima cat (*Felis bengalensis euptilura*), an endangered Japanese nondomestic subspecies of leopard cat (*F. bengalensis*). Phylogenetic analysis of the *env* gene sequences indicated that the FIV from the Tsushima cat belonged to a cluster of subtype D FIVs from domestic cats. FIVs from both the Tsushima cat and the domestic cat showed similar levels of replication and cytopathicity in lymphoid cell lines derived from these two species. The results indicated the occurrence of interspecies transmission of FIV from the domestic cat to the Tsushima cat in the wild.

Feline immunodeficiency virus (FIV) is a causative agent of AIDS-like disease and immunological abnormalities in domestic cats (*Felis catus*) that are similar to those caused by the human immunodeficiency virus (HIV) in humans (1, 25, 26). FIV isolates have been classified into five distinct subtypes: A, B, C, D, and E (10, 16, 21–24). Subtypes B and D are the two major subtypes that are distributed throughout the northeastern and southwestern districts of Japan (16). Other FIV-related lentiviruses detected in nondomestic wild cats, including the puma (*Puma concolour*), lion (*Panthera leo*), and Pallas's cat (*F. manul*), are known to be distantly related to FIVs of domestic cats by phylogenetic analyses (2–4, 11, 20).

A Tsushima cat (*Felis bengalensis euptilura*) that was captured in the forest of Tsushima Island for the purpose of breeding in a zoo was found to be positive for FIV antibody. The Tsushima cat is an endangered wild cat discovered in Japan in recent years, a local form of the Siberian subspecies of the leopard cat (*F. bengalensis*). It inhabits busy forest edges and paddy fields near the coast of Tsushima Island off the Japanese mainland. It is larger than the continental subspecies but smaller than another Japanese subspecies (*F. bengalensis iriomotensis*). Its population size is between 70 and 90 individuals. Therefore, this subspecies of leopard cats seems to be a relic population that separated from other leopard cats long ago (14).

In this study, the Tsushima cat virus was analyzed phylogenetically to help explain the origin and transmission of FIV or FIV-related lentiviruses. Furthermore, the growth and cytopathicity of the virus from the Tsushima cat and FIV from the domestic cat were examined in lymphoid cell lines from both species.

Virus isolation from the FIV-positive Tsushima cat. At the serum examinations immediately upon capture, the wild-caught Tsushima cat was positive for FIV antibody and negative for feline leukemia virus (FeLV) antigen in serological tests using a commercial test kit (SnapTM; IDEXX, Portland, Maine). After capture, the Tsushima cat was kept in a special facility in Fukuoka Municipal Zoo and Botanic Garden, completely isolated from any other animals, including domestic cats. Hematological examination of the Tsushima cat revealed a lymphocyte count of 1,480/μl, which was within the range of four previously examined healthy Tsushima cats (1,400 to 3,000/μl). A $CD8^+$ cell count, determined by using a monoclonal antibody against cat lymphocytes (Southern Biotechnology, Birmingham, Ala.), for the Tsushima cat was 435/μl, which was similar to the values in previously examined healthy Tsushima cats. A $CD4^+$ cell count could not be determined because a monoclonal antibody against feline CD4 (Southern Biotechnology) did not react with lymphocytes of the Tsushima cat.

For virus isolation, a Tsushima cat-derived lymphoid cell line, PIPP-I, was established from a zoo-kept Tsushima cat that was seronegative for FIV, FeLV, and other common pathogens in domestic cats. The cytochrome *b* sequence from the PIPP-I cell line was amplified by PCR with primers described previously (14), and the sequence indicated that the cell line originated from a Tsushima cat. The PIPP-I cell line stained positively with monoclonal antibodies against CD8, CD9 (MM2/57; Southern Biotechnology), and interleukin-2 receptor alpha (9F23) in flow cytometric analyses (18). A feline $CD4^+$ T-lymphoid cell line, Kumi-1 (7), was also used for virus isolation. Peripheral blood mononuclear cells (PBMC) were obtained from the seropositive Tsushima cat. The cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum and were stimulated with 10 μg of concanavalin A per ml for 3 days in the presence of 100 U of human

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recombinant interleukin-2 (Pharma Biotechnologie, Hannover, Germany) per ml. The Tsushima cat lymphoid cells were cocultivated with PIPP-I cells or Kumi-1 cells 30 days after initiation of the culture. Reverse transcriptase (RT) assay as described previously (19) showed an increase in Mg-dependent RT activities in the culture supernatants of cocultures of PIPP-I and Kumi-1 cells by 20 to 30 days after initiation of cocultivation. The RT-positive culture supernatants from the lymphocyte cocultures of PIPP-I cells and Kumi-1 cells were frozen at −80°C as virus stocks designated Feu-P and Feu-K, respectively.

Sequence analyses of viruses isolated from the Tsushima cat and FIVs from domestic cats from Tsushima Island. For the analysis of proviral DNA of FIV or FIV-related lentivirus, high-molecular-weight DNAs were extracted from primary PBMC that were obtained from the Tsushima cat seropositive for FIV antibody and the cocultures with either PIPP-I or Kumi-1 cells. These DNA samples were used for nested PCR amplification of the FIV *env* gene spanning regions V3 to V6. The nested PCR primers used in this study were described in our previous paper (17). PCR products were directly cloned into a cloning vector (TA cloning kit; Invitrogen, San Diego, Calif.) and sequenced by using the dideoxy chain termination method. Alignments of the deduced amino acid sequences of 9 viral genomes obtained from the primary PBMC (Feu1, -2, and -3), coculture with PIPP-I cells (Feu4, -5, and -6), and coculture with Kumi-1 cells (Feu7, -8 and -9) were almost identical, showing only 1 to 23 nucleotide substitutions in the 624-bp fragment. The proviral sequences from the Tsushima cat showed relatively high amino acid sequence similarities (82.7 to 93.8%) with those of subtype D FIV strains previously reported and lower sequence similarities with those of subtype A, B, and C strains of FIV (71.6 to 80.7%) in Japan (10, 16, 17) (Fig. 1).

Furthermore, for the purpose of the investigation of the origin and transmission of FIV, blood samples were collected from 50 stray domestic cats from two villages on Tsushima Island, A and B, near the forest where the seropositive Tsushima cat was captured. Serum samples from the domestic cats showed a high frequency (11 of 51, or 21.6%) of positive results for antibodies against FIV. From the primary PBMC samples obtained from 10 domestic cats seropositive for FIV antibody, the proviral sequences of FIV were sequenced after nested PCR amplification of the *env* gene fragment. All 10 strains obtained from the domestic cats from Tsushima Island (TSU101, TSU102, TSU104, TSU107, TSU109, and TSU116 strains obtained in village A and TSU202, TSU210, TSU215, TSU226, and TSU226 strains obtained in village B) had sequences that were highly similar to those of subtype D FIVs as well as to those from the Tsushima cat obtained in this study (Fig. 1). To rule out the possibility of contamination by PCR products, we carried out three independent PCR amplifications for each of the DNA templates obtained from Tsushima cat-derived and domestic cat-derived samples and obtained almost the same results. Control PCR amplification without template DNA did not generate any amplified product.

Phylogenetic analyses for the proviral DNA sequences. Nucleotide divergences for pairs of sequences were estimated by using the neighbor-joining method in the DNADIST program from the PHYLIP software package (5). A phylogenetic tree constructed with the nucleotide sequences spanning the V3 to the V5 region of the FIV *env* gene was constructed by using the NEIGHBOR program, and the branching order reliability was evaluated by using bootstrap analysis in the SEQBOOT program (5). All of the proviral DNA sequences from the Tsushima cat (Feu1 to -9), from domestic cats in village A (TSU101, TSU102, TSU104, TSU107, TSU109, and TSU116), and those in village B (TSU202, TSU210, TSU215, and TSU226) belonged to the subtype D FIVs of domestic cats, with high bootstrap values (Fig. 2). Feu1 to -3 clones obtained from the Tsushima cat primary PBMC and Feu4 to -9 clones obtained from cocultures with PIPP-I and Kumi-1 cells clustered in the subtype D FIVs (Fig. 2). Most of the isolates obtained from domestic cats living in village A (TSU101, TSU104, TSU107, TSU109, and TSU116) formed another cluster. On the basis of the phylogenetic analysis, FIV could have been transmitted to the Tsushima cat from any subtype D-infected domestic cat. A maximum likelihood phylogenetic tree constructed by using the DNAML program (5) supported the branching orders, showing high bootstrap values (>70) in the unrooted neighbor-joining phylogenetic tree, with significant P values ($P < 0.01$) (data not shown).

Replication and cytopathicity of the isolates from the Tsushima cat and the domestic cat. To investigate the interspecies transmission of FIV, we examined the replication and cytopathicity of the FIVs from the Tsushima cat and from domestic cats in lymphoid cell lines from these two species. Feu-P and Feu-K strains in the cocultures with PIPP-I and Kumi-1 cells, respectively, were used as virus stocks of FIV from the Tsushima cat. A strain isolated from a domestic cat in Fukuoka in southwestern Japan (7) for use as an FIV domestic-cat stock virus was designated reference subtype D FIV. PIPP-I cells (10^6 cells/ml) in a 6-well culture plate were inoculated with an equivalent number of virus particles (RT activity, 2,000 cpm) from the various FIV isolates. After adsorption at 37°C for 1 h, the cells were washed with phosphate-buffered saline and cultured in fresh RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were cultured and maintained at 37°C with medium changes every third day. RT activity in the culture supernatants and the number of viable cells counted using trypan blue staining were monitored after virus inoculation. All experiments were performed in duplicate. To rule out the possibility of contamination, we carried out the sequencing of the proviruses from the cultured cells infected with these viral strains at the end of the cultures and confirmed that there was no laboratory contamination of the virus strains used in this study. In the PIPP-I cells, both Tsushima cat-derived viruses (Feu-P and Feu-K) and domestic cat-derived virus (Fukuoka) displayed similar growth rates as shown by an increase in culture supernatant RT starting at 6 days after inoculation, reaching the highest levels (1×10^6 to 1.5×10^6 cpm/ml) at 12 to 18 days after inoculation (Fig. 3A). The viable cell counts of PIPP-I cells progressively decreased in contrast to the increase in RT activity and finally decreased to as few as 2×10^5 cells/ml (Fig. 3B). A proportion of the PIPP-I cells producing a large amount of FIV showed shrinkage of the cells and condensation of the nuclear chromatin, which are features characteristic of apoptosis. The growth curve of the viruses and the number of viable cell counts in PIPP-I cells after infection revealed no obvious differences in these parameters between the Tsushima cat-derived and domestic cat-derived viruses. In the Kumi-1 cells from the domestic cat, viral growth rate and cytopathicity were essentially similar to those obtained with PIPP-I cells (data not shown). The results indicated that the viruses from the Tsushima cat and the domestic cat shared similar growth curves and cytopathicities in Tsushima cat and domestic cat lymphoid cells.

The seropositivity of FIV antibodies in stray domestic cats from Tsushima Island was found to be very high, 21.6%, relative to those in mainland Japan and other countries (6, 8, 9, 13, 29). All of the FIV proviral genomes obtained from domestic cats of Tsushima Island belonged to subtype D, which is the most common subtype on the nearby much larger island of

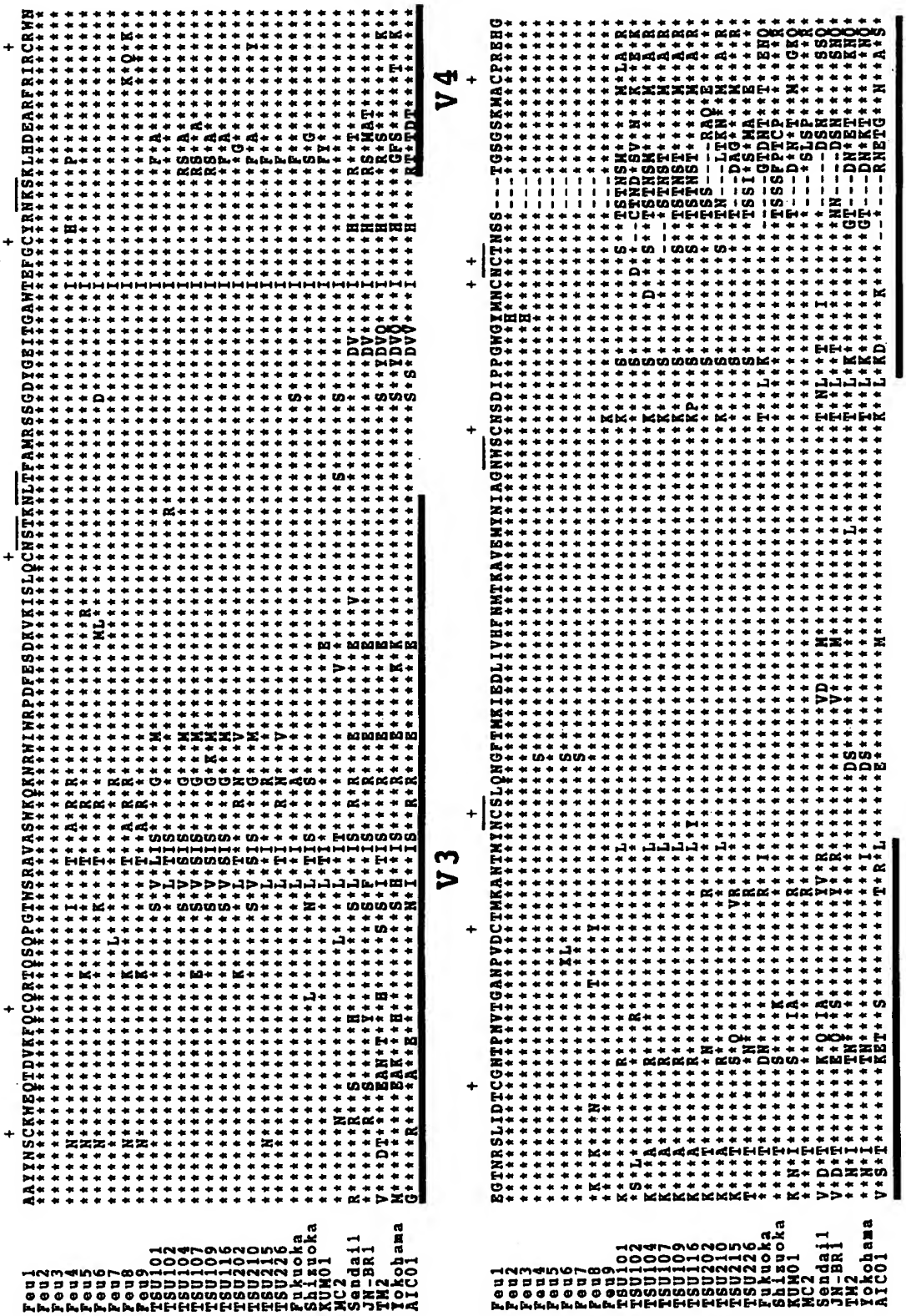

FIG. 1. Alignment of the predicted amino acid sequences of the FIV *env* gene of 9 clones from a Tsushima cat (Feu1 to -9), 10 clones from domestic cats on Tsushima Island (TSU101, TSU102, TSU104, TSU107, TSU109, TSU116, TSU202, TSU210, TSU215, and TSU226), and 9 known FIV clones, by using the CLUSTAL W program. The FIV clones previously reported include Fukuoka (GenBank/EMBL/DDBJ accession no. D37815), Shizuoka (D37811), KUM01 (AB010405), MC2 (D67062), Sendai1 (D37813), JN-BR1 (D67052), TM2 (M59418), Yokohama (D37812), and AIC01 (AB010395). Asterisks denote amino acid identity with the Feu1 strain. The horizontal lines above the sequence indicate the locations of N-linked glycosylation sites. The pluses indicate the positions of cysteine residues. The dashes represent gaps introduced for optimal alignment. The thick bars under the alignment indicate the positions of the variable regions V3 to V5 as defined by Pancino et al. (21).

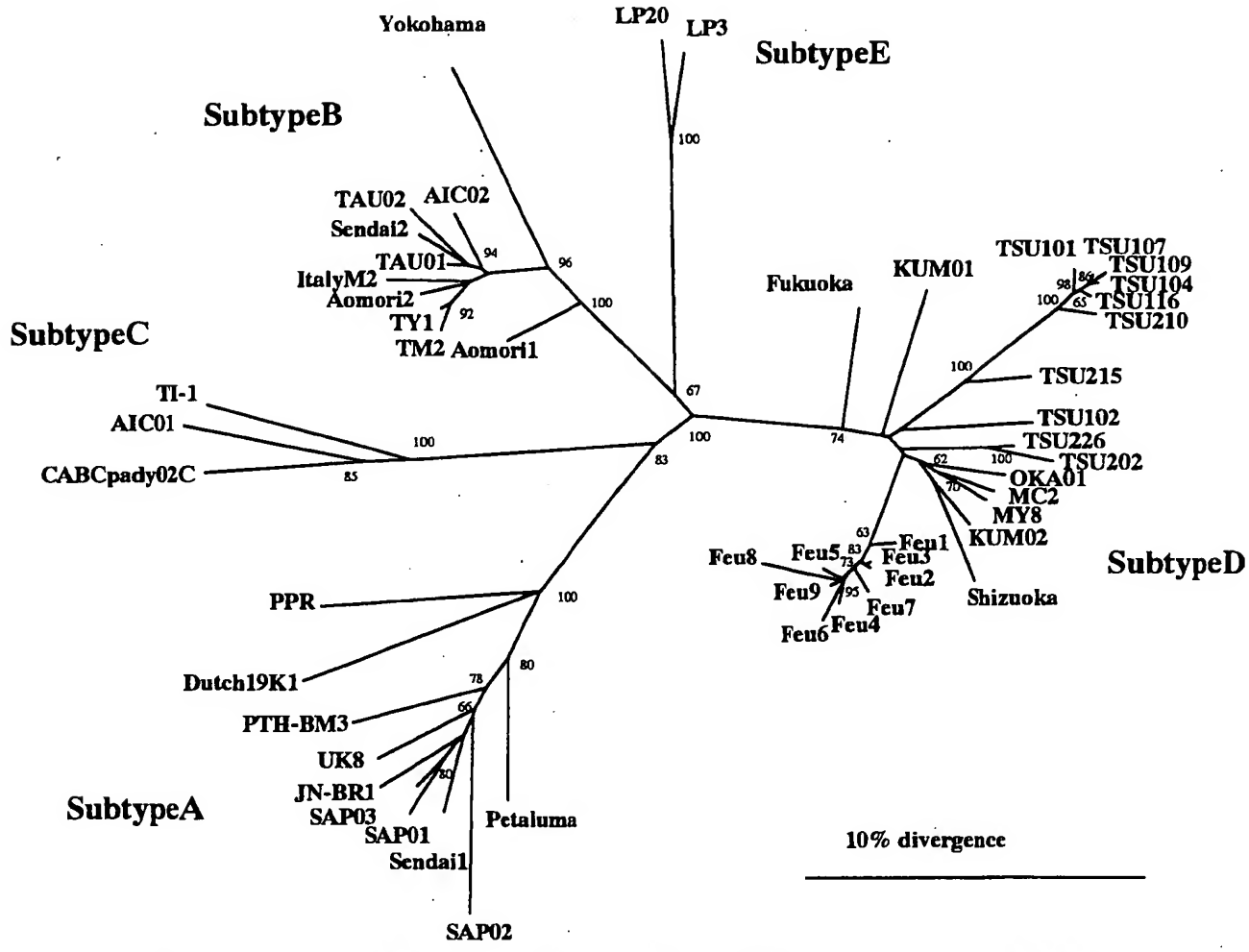

FIG. 2. An unrooted neighbor-joining phylogenetic tree of the FIV *env* gene covering variable regions V3 to V5. Nucleotide divergences were estimated by the DNADIST program from the PHYLIP software package (5). The phylogenic tree was constructed by using the NEIGHBOR program, and the branching order reliability was evaluated by bootstrap analysis in the SEQBOOT program (5). Virus clones obtained in this study are Feu1 to -9 from the Tsushima cat and TSU101, TSU102, TSU104, TSU107, TSU109, TSU116, TSU202, TSU210, TSU215, and TSU226 from domestic cats on Tsushima Island. The FIV clones previously reported are Petaluma FIV14 (GenBank/EMBL/DDBJ accession no. M25381), TM2 (M59418), Yokohama (D37812), Shizuoka (D37811), JN-BR1 (D67052), MC2 (D67062), MY8 (D67063), TY1 (D67064), Sendai1 (D37813), Sendai2 (D37814), Aomori1 (D37816), Aomori2 (D37817), Fukuoka (D37815), PPR (M36968), UK8 (X69496), Dutch19k1 (M73964), ItalyM2 (X69501), LP3 (D84496), LP20 (D84498), TI-1, CABCpady02C (U02392), SAP01 (AB010402), SAP02 (AB010403), SAP03 (AB010404), PTH-BM3 (AB010401), TAU01 (AB10405), TAU02 (AB10406), AIC01 (AB10396), AIC02 (AB10397), OKA01 (AB010400), KUM01 (AB010398), and KUM02 (AB010399). The numbers at each branch point indicate the bootstrap values (5) preserved through greater than 60 in 100 bootstrap repetitions.

Kyushu. The subtype D viruses endemic on Tsushima Island were assumed, therefore, to have been introduced from domestic cats from Kyushu. Though it is not clear when the subtype D FIV was introduced to Tsushima Island, there have been close relations via traffic by sea and air for a long time between the people living on Kyushu and those living on Tsushima Island. Therefore, domestic cats infected with subtype D FIV endemic in Kyushu could be easily introduced to Tsushima Island. The fact that there are only two species belonging to the *Felidae* family would support the theory that FIV could have been transmitted to the Tsushima cat from any subtype D-infected domestic cat. We found that 16 wild-caught Tsushima cats other than the animals in this study were seronegative for FIV antibody, indicating that FIV infection is not common in the Tsushima cat. Moreover, many species belonging to the *Felidae* family were reported to be infected with lentiviruses that were different from each other. The areas where each species lives are isolated from other species. It is conceivable that the FIV-infected Tsushima cat found in this study might have accidentally come into contact with a seropositive domestic cat in the forest or adjacent villages; recent deforestation and development on Tsushima Island have eroded the forests that Tsushima cats inhabit, making this scenario probable. A large part of the conservation of the rare Tsushima cat may rest with conservation of its environment, which serves as an important barrier to contact between this highly endangered species and infectious domestic cats.

Domestic cats can be infected with puma and lion lentiviruses; however, the inocula used to demonstrate these infections were viruses isolated by cocultivation with domestic cat PBMC and the cat lymphoid cell line 3201 (27, 28). In general, adaptation of virus for other host species has been shown to be an important factor for interspecies transmission. However, in the present study, FIVs from both the Tsushima cat and domestic cats showed similar replicative capacities in both PIPP-I and Kumi-1 cell types, indicating that interspecies transmission

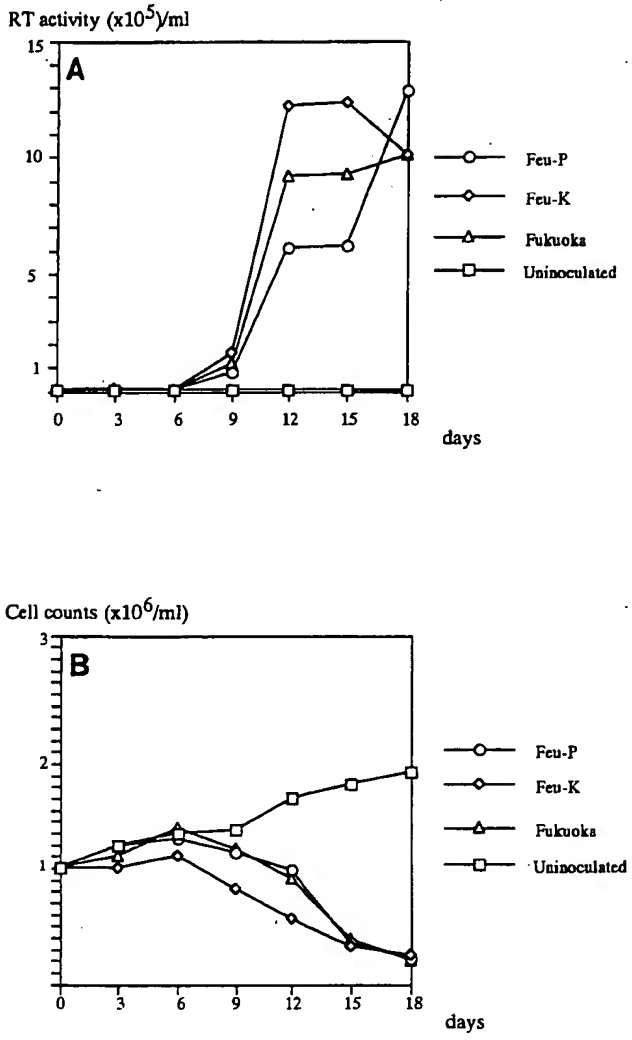

FIG. 3. Replication of the FIV isolates and the viable cell counts in PIPP-I cells after infection with the FIV isolates. (A) Production of FIV as examined by RT assay of the culture supernatants from PIPP-I cells. (B) Counts of viable PIPP-I cells. Feu-P, Tsushima cat-derived FIV isolated by cocultivation with PIPP-I cells; Feu-K, Tsushima cat-derived FIV isolated by cocultivation with Kumi-1 cells; Fukuoka, domestic cat-derived FIV isolated by cocultivation with Kumi-1 cells.

of FIV between domestic cats and Tsushima cats was highly likely. FIV-related lentiviruses in the puma and lion apparently do not induce any symptomatic disease in their natural host or in experimentally infected domestic cats (27). The case is different with simian immunodeficiency virus (SIV) infection; SIV from sooty mangabeys induced an AIDS-like disease in Asian macaques, though the virus did not cause any disease in its natural host (12, 15). Our data indicated that FIV from the domestic cat was cytopathic in cells cultured from the lymphoid cells of Tsushima cats, yet we presently do not know whether FIV can induce disease in the Tsushima cat; continued observation of possible disease progression in this FIV-infected Tsushima cat should eventually reveal the link, or lack of a link, between this virus and disease in this host.

The present study is an example of interspecies transmission of a pathogen from the domestic cat to an endangered non-domestic cat species in the wild. For the conservation of a diversity of wild animal species, it will be important to control the threat to endangered species from diseases carried by related domestic species.

Nucleotide sequence accession numbers. Nucleotide sequences of the FIV genome obtained in this study have been deposited in the DDBJ database under accession no. AB02110 through AB02118.

We are grateful to H. Koyama and T. Hohdatsu (Kitasato University) for providing the FIV Fukuoka strain and Kumi-1 cell line.

This study was supported by the Environment Agency of Japan, the Japan Health Sciences Foundation, and grants from the Ministry of Education, Science, Sports and Culture and the Ministry of Health and Welfare of Japan.

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Feline leukemia virus infection in a captive cheetah and the clinical and antibody response of six captive cheetahs to vaccination with a subunit feline leukemia virus vaccine

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IN 1982 AND 1983, feline infectious peritonitis (FIP) was diagnosed in a group of 35 cheetahs in a 6.1-ha fenced enclosure at Wildlife Safari in Winston, Ore.[1,2] On Aug 10, 1984 (day 1), one of the 21 remaining cheetahs in this group, a 3.5-year-old female cheetah (No. 1), was reported by a keeper to be depressed and anorectic. Initial examination indicated that the nictitating membranes of the cheetah were protruded and that the cheetah appeared to be underweight and dehydrated. Parasite ova were not found on a fecal flotation examination. The following day (day 2), the cheetah was anesthetized with a combination of ketamine HCl (300 mg) and xylazine (10 mg) administered IM via a blowdart. Examination indicated that the cheetah had a normal rectal temperature (39 C), pulse rate (100/min), and respiratory rate (16/min) and weighed 32 kg, which was considered to be 8 kg underweight. The cheetah was estimated to be 5% dehydrated, on the basis of skin elasticity, as compared with that of healthy cheetahs.

Urinalysis did not indicate abnormalities. Blood analyses indicated a WBC count of 15,000 cells/μl, with a differential count of 80% segmented neutrophils, 19% lymphocytes, and 1% monocytes. Erythrocyte indices indicated that the cheetah had large erythrocytes (mean corpuscular volume, 74 fl [normal = 39 to 55 fl]), but normal hemoglobin content (mean corpuscular hemoglobin content, 30.3%). The PCV (45.1%) and the plasma protein concentration (9.2 g/dl) were high, probably because of the cheetah's dehydration. Moderate anisocytosis and polychromasia were found on blood smears. Serum samples were weakly positive for feline coronavirus antibody (titer = 1:25; indirect immunofluorescent antibody test)[3,a] and were positive for feline leukemia virus (FeLV) antigen (enzyme-linked immunosorbent assay [ELISA]).[b,c]

The cheetah was given lactated Ringer's solution (500 ml) IV and multi-B complex vitamins[d] (2 ml) IM daily for one week. Because tapeworms had been a problem in the group of cheetahs, the cheetah was given praziquantel[e] (2.6 ml) once SC. Because of the positive FeLV test, the cheetah was isolated from the remainder of the group. Amoxicillin (500 mg, orally) was given once daily for 14 days. Because corticosteroids may be beneficial in the treatment of some myeloproliferative disorders associated with FeLV infections,[4,5] the cheetah was given prednisolone orally (50 mg daily for one week, 50 mg every third day for 2 weeks, and 25 mg every third day for 2 weeks). During this 5-week period of prednisolone treatment, the cheetah's appetite returned to normal.

At the end of the 5-week treatment period, the cheetah was anesthetized and examined, weighed 40 kg, and appeared to be normally hydrated. Although the BUN concentration was high (81 mg/dl) and the leukocytosis persisted (22,000 cells/μl), the other blood values were normal, as compared with those of healthy cheetahs in the group. The serum remained slightly positive for FeLV antigen; therefore, the cheetah remained in isolation. The FeLV tests were repeated monthly thereafter. The next 3 FeLV tests were negative for FeLV antigen; therefore, the cheetah was returned to the group. Recurrence of illness did not develop (20 months).

Feline leukemia virus infection was diagnosed on the basis of positive FeLV tests, the nonspecific clinical signs, and retrospective serologic findings in the other cheetahs in the group. Two other cheetahs in the group (cheetahs 2 and 3) had been positive for FeLV antigen by ELISA 1 to 3 months before the development of illness in cheetah 1, and 2 other cheetahs (No. 4 and 8) had suspicious reactions for FeLV antigen by ELISA. Cheetahs 2, 3, 4, and 8 did not develop clinical signs of illness and were negative for FeLV antigen on subsequent ELISA before the occurrence of the problems observed in cheetah 1. Although not conclusive, detection of FeLV antigen in cheetahs 2 and 3, and possibly in cheetahs 4 and 8, indicated that several cheetahs in the group associated with cheetah 1 may have been exposed previously to FeLV. The previous diagnosis of FIP in cheetahs of this group[1,2] also was suggestive of a possible FeLV infection among cheetahs in this preserve.[6-8]

Evaluation of cheetahs vaccinated with a subunit FeLV vaccine—seventeen of the 21 cheetahs (No. 3 through 19) were anesthesized with a combination of ketamine HCl (7 mg/kg of body weight) and xylazine (5 mg/animal) administered IM. Blood (20 ml) was collected from each cheetah into clot tubes. Serum samples (1 ml/cheetah) were evaluated for FeLV anti-

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The authors thank Dr. Richard Sharpee for conducting the antibody studies and Norden Laboratories, Lincoln, Neb, for supplying the vaccine used.

[a]Washington Animal Disease Diagnostic Laboratory, Pullman, Wash.
[b]Parkway Animal Hospital, Roseburg, Ore.
[c]Leukassay F, Pitman-Moore Inc, Washington Crossing, NJ.
[d]Combiplex-B, Tech American Group Inc, Elwood, Kan.
[e]Droncit Injectable, Bayvet Corp, Division of Miles Laboratories, Shawnee Mission, Kan.

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gen by use of ELISA. The remaining serum was frozen (−70 C) for future evaluations. The 17 cheetahs were negative for FeLV antigen. Blood was not collected from the affected cheetah (No. 1) at this time, nor was blood collected from 3 cheetahs that were isolated from this group.

The 17 FeLV-negative cheetahs were each given 2 ml of a subunit feline leukemia vaccine[f] IM. A second 2-ml dose was administered 2 to 3 weeks after the first vaccination. Fifteen of the 17 cheetahs were given a third 2-ml dose 2 months after the second dose. Before receiving the third dose of vaccine, one cheetah (No. 4) died of renal failure that was not associated with vaccination, and another cheetah (No. 18) that had been on loan was returned to the owner.

Adverse reactions to the vaccine were minimal. After the first dose of vaccine, 2 cheetahs (No. 5 and 11) developed mild to moderate facial swelling in the areas surrounding the mandibular lymph nodes; these reactions were not severe enough to restrict respiration or ingestion of food or water. After the second dose of vaccine, 2 other cheetahs (No. 6 and 12) developed similar facial swelling around the mandibular lymph nodes. Another cheetah (No. 4) became moderately depressed for 3 to 4 days after the second vaccination. Adverse reactions did not develop in the 15 remaining cheetahs after the third dose of vaccine. Although injection sites were not palpated, swelling or erythema was not seen nor was lameness evident.

Approximately 3 weeks after the third vaccination, 6 of the 15 vaccinated cheetahs (No. 5 through 10) were selected randomly and anesthetized with ketamine HCl and xylazine. Blood was collected, and the serum samples were frozen (−70 C) until determination of antibody titers against feline oncornavirus cell membrane antigen (FOCMA), using an immunofluorescent procedure with FL74 lymphoblastoid cells as the target,[g] and until determination of antibodies against FeLV envelope antigen (gp70), using an ELISA.[g] Compared with prevaccination titers, titers against FOCMA and gp70 markedly increased after vaccination (Table 1). Prevaccination titers against FOCMA were 1:2 to 1:4 (titers of <1:8 are considered negative), whereas postvaccination titers were 1:16 to 1:256. Prevaccination titers against gp70 were 0.037 to 0.067 optic density (OD; values <0.1 OD were considered negative), whereas postvaccination titers were 0.224 to 1.053 OD.

TABLE 1—Antibody responses of 6 cheetahs before and after vaccination with a subunit feline leukemia virus vaccine

Cheetah No.	Sex	Age	Blood sample*	Antibody titers: gp70 (OD)†	Antibody titers: Anti-FOCMA‡
5	Male	Adult	Pre	0.067	1:4
			Post	0.364	1:16
6	Male	3 yrs	Pre	0.042	1:4
			Post	0.933	1:256
7	Female	Adult	Pre	0.039	1:4
			Post	1.033	1:64
8	Male	Adult	Pre	0.054	1:4
			Post	0.768	1:64
9	Female	Adult	Pre	0.059	1:4
			Post	1.053	1:32
10	Male	Adult	Pre	0.037	1:2
			Post	0.224	1:16

*Pre = prevaccination sample; Post = postvaccination sample.
†Results are reported as optic density (OD). Values >0.1 are considered to be positive for antibody against the FeLV envelope antigen (gp70). ‡Titers >1:8 are considered positive for antibody against feline oncornavirus cell membrane antigen (FOCMA)

Discussion

Feline leukemia virus causes severe illness and death in domestic cats; however, FeLV infection and the so-called FeLV-related diseases rarely have been reported in exotic feline species.[9–13] In domestic cats, 46% to 62% of cats with FIP are infected with FeLV.[6–8] Feline infectious peritonitis also has been reported in exotic felids.[1,2,14] Perhaps, FeLV infections are more prevalent in exotic felids than has been recognized previously.

Feline leukemia virus infection or FeLV-related diseases have not been detected in the cheetahs on the preserve since completion of the vaccination program. Whether the serologic responses seen in the vaccinates were a result of the vaccine or possibly due to exposure to FeLV could not be determined conclusively; however, the results of the present report indicate that the responses probably were vaccine-induced. Blood samples were not collected from the nonvaccinated cheetahs because of isolation of these cheetahs, the value of these cheetahs, and the fact that these cheetahs were pregnant or nursing kittens at the time that blood samples were collected from the other cheetahs. Therefore, serologic comparison between the vaccinated and nonvaccinated cheetahs was not done.

The data indicated that vaccination of cheetahs with the FeLV vaccine used in the present report probably is a safe procedure in that the vaccine did not cause detectable ill effects in the 17 cheetahs vaccinated. Vaccination of 6 cheetahs resulted in a marked increase in antibody titers against FOCMA and the gp70 antigen. If these titers are protective against FeLV infection, vaccination of cheetahs against FeLV may be warranted in captive cheetah populations in which FeLV infections are suspected. Such a vaccination program should be combined with evaluations for FeLV and isolation of FeLV-positive animals.

[f]Leukocell. Norden Laboratories. Lincoln, Neb.
[g]Norden Laboratories. Lincoln, Neb.

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Vertebral body fracture in a captive cheetah

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AN 11-YEAR-OLD male cheetah developed acute paraparesis and ataxia 10 months after arriving at the St Louis Zoo. The cheetah appeared alert, with normal thoracic limb function. The pelvic limbs were bilaterally and asymmetrically paretic. The left pelvic limb had more prominent deficits than did the right pelvic limb. Gait abnormalities were mild swaying to intermittent, abrupt collapse in the hindquarters. The cat arched its back in exaggerated dorsoflexion when attempting to hunch over its food dish in a typical feeding posture.

The cheetah was anesthetized with ketamine HCl[a] (11.35 mg/kg of body weight, IM) and xylazine HCl[b] (1.17 mg/kg, IM), which were administered by use of a blowdart.[c] Halothane was used to maintain anesthesia. The CBC and serum biochemical values were normal.[1] Serum titers against feline leukemia virus and cheetah coronavirus were not detected. Physical examination did not indicate evidence of primary myocardial disease (ie, cardiac auscultation was normal, the femoral pulses were strong, and the pelvic limbs were warm to the touch[2]).

Initial survey radiography indicated a possible intervertebral disk extrusion (ie, narrowing of the L4-L5 spaces[3,4]). Close examination of the radiographs indicated a bony exostosis in association with the lateral rim of L5. This subtle lesion was assumed to be an age-related change and of minimal clinical importance. The sharp margins of the involved vertebral bodies discounted the likelihood of a chronic disease process. An acute traumatic lesion of the spinal cord, resulting in an uncomplicated disk extrusion, was suspected; however, the possibility of other primary vertebral or spinal cord lesions could not be discounted.

The cheetah was reevaluated after 11 weeks of strict confinement and physical inactivity. The persistence of a stiffened, protected gait prompted

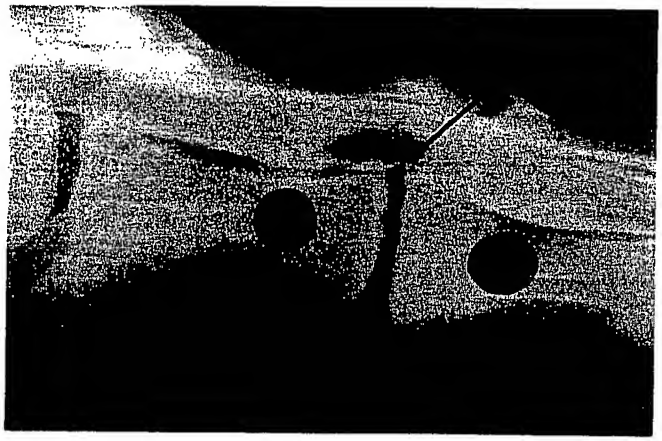

Fig 1—Myelograph of the L4-L5 region of a cheetah. The arrow points to a subtle narrowing of contrast material at the dorsocranial edge of L5. Notice the slight narrowing between L4 and L5, as compared with the cranial space between L3 and L4.

Fig 2—Computerized tomography of a cheetah at the level of L5. The asymmetry and space deficit (arrow) formed by a displaced fracture fragment impinging on the anterior aspect of the cord.

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The authors thank Drs. Everett Aronson and Dennis O'Brien, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211.

[a]Ketaset, Bristol Laboratories, Syracuse, NY.
[b]Rompun, Haver-Lockhart, Bayvet, Shawnee, Kan.
[c]Telinject USA, Newhall, Calif.

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Use of a subunit feline leukemia virus vaccine in exotic cats.

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Three adult bengal tigers, 2 immature white tigers, and 3 adult servals were vaccinated IM with three 1-ml doses of a subunit FeLV vaccine with dosage interval guidelines of the manufacturer. All cats had increased antibody titers FeLV gp 70 capsular antigen and feline oncornavirus cell membrane-associa antigen during the vaccination trial. Three weeks after the third vaccination, 8 cats had gp70 antibody titers greater than 0.2 (optical density), and all 8 cat feline oncornavirus cell membrane-associated antigen antibody titers greater 1:8.

PMID: 2835347 [PubMed - indexed for MEDLINE]

JOURNAL OF VIROLOGY, Sept. 1994, p. 5953–5968
0022-538X/94/$04.00+0

Vol. 68, No. 9

A Lion Lentivirus Related to Feline Immunodeficiency Virus: Epidemiologic and Phylogenetic Aspects

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Received 23 February 1994/Accepted 16 June 1994

Feline immunodeficiency virus (FIV) is a novel lentivirus that is genetically homologous and functionally analogous to the human AIDS viruses, human immunodeficiency virus types 1 and 2. FIV causes immunosuppression in domestic cats by destroying the CD4 T-lymphocyte subsets in infected hosts. A serological survey of over 400 free-ranging African and Asian lions (*Panthera leo*) for antibodies to FIV revealed endemic lentivirus prevalence with an incidence of seropositivity as high as 90%. A lion lentivirus (FIV-Ple) was isolated by infection of lion lymphocytes in vitro. Seroconversion was documented in two Serengeti lions, and discordance of mother-cub serological status argues against maternal transmission (in favor of horizontal spread) as a major route of infection among lions. A phylogenetic analysis of cloned FIV-Ple *pol* gene sequences from 27 lions from four African populations (from the Serengeti reserve, Ngorongoro Crater, Lake Manyara, and Kruger Park) revealed remarkably high intra- and interindividual genetic diversity at the sequence level. Three FIV-Ple phylogenetic clusters or clades were resolved with phenetic, parsimony, and likelihood analytical procedures. The three clades, which occurred not only together in the same population but throughout Africa, were as divergent from each other as were homologous *pol* sequences of lentivirus isolated from distinct feline species, i.e., puma and domestic cat. The FIV-Ple clades, however, were more closely related to each other than to other feline lentiviruses (monophyletic for lion species), suggesting that the ancestors of FIV-Ple evolved in allopatric (geographically isolated) lion populations that converged recently. To date, there is no clear evidence of FIV-Ple-associated pathology, raising the possibility of a historic genetic accommodation of the lion lentivirus and its host leading to a coevolved host-parasite symbiosis (or commensalism) in the population similar to that hypothesized for endemic simian immunodeficiency virus without pathology in free-ranging African monkey species.

The global increase of human immunodeficiency virus (HIV) infection over the past decade has intensified efforts to understand the phylogenetic origins and population dynamics of the HIV types 1 and 2 (HIV-1 and HIV-2) lentiviruses, the causative agent of AIDS. Lentivirus infection in humans is thought to have emerged from a recent transspecies infection of an ancestral lentivirus that may previously have been harbored in two groups of nonhuman primates (2, 10, 17, 35). This inference is supported by the isolation of the simian immunodeficiency virus (SIV) SIV-CPZ, related to HIV-1, from a chimpanzee in Gabon and by the occurrence of SIV-SM, related to HIV-2, in sooty mangabeys (10, 19, 35, 48). SIVs from *Cercopithecus* species, African green monkeys, display extensive inter se genetic divergence, suggesting that SIV infection in nonhuman African primates may be an ancient event (21, 37).

Lentiviruses are highly unstable RNA viruses. The high error rates of the viral polymerase in DNA synthesis result in the rapid evolution of genetic variants following initial infection of the host organism (12, 18, 22, 46). These evolving retroviral variants, or quasispecies, continue gathering mutations in vivo, resulting in the maintenance of a genetically diverse retroviral population throughout the term of viremia (4, 13). Quasispecies have been observed to diverge as much as 5% across the proviral genome and are capable of divergent and convergent evolutionary schemes (3, 20). The differentiation of HIV quasispecies may ultimately lead to the formation of "escape variants," pathological genomic mutants capable of avoiding host immune surveillance and possibly of enhancing disease progression (31, 43).

Lentiviruses closely related to HIV have been isolated from monkeys, goats, sheep, horses, cattle, and cats (15, 17, 23, 36, 37, 48, 49). The feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus that causes immune suppression by gradually depleting the CD4 T-cell subsets in domestic cats (*Felis catus*) (1, 42, 55). FIV infection of the domestic cat provides a useful small animal model for the study of HIV infection in humans (44, 46). Like HIV infection of humans, FIV causes gradual dysfunction of the feline immune system which results in immune deficiency and opportunistic infections in infected hosts (42, 55). Although other transmission modes have not been adequately explored, FIV appears to be spread via the saliva during biting (54, 55). Antibodies to FIV have been detected in several nondomestic felid species, including cheetahs, pumas, bobcats, jaguars, and leopards (5, 7, 8, 25, 37).

East African lions (*Panthera leo*) inhabit the Serengeti reserve and Ngorongoro Crater in Tanzania. Although these lion habitats are contiguous in East Africa, the two populations reflect quite different natural histories. The Ngorongoro Crater lions are known to have undergone a series of population bottlenecks that had previously reduced the Crater population to as few as 10 individuals (29, 32, 41, 53). In contrast, the Serengeti plains and woodlands harbor a large outbred popu-

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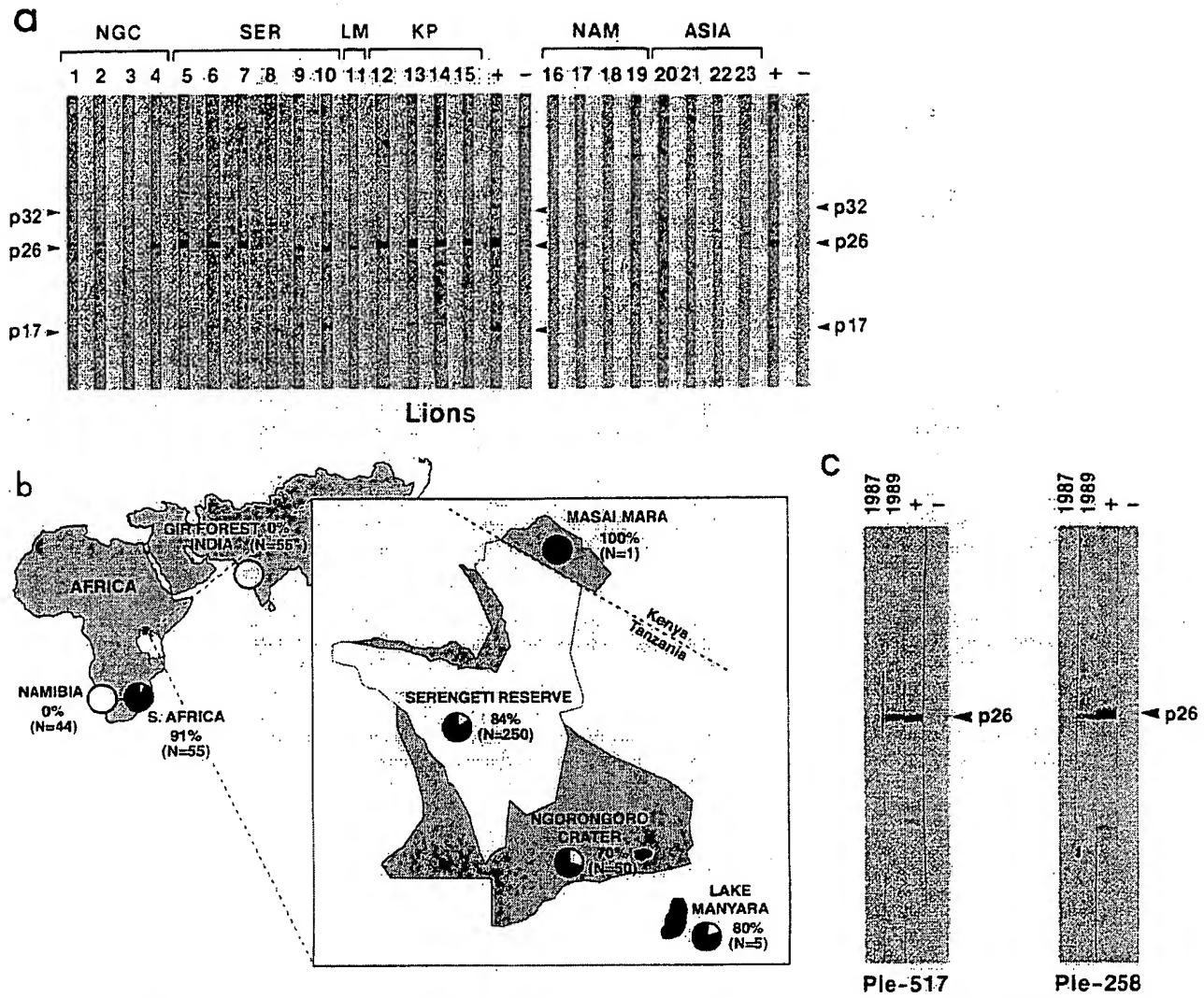
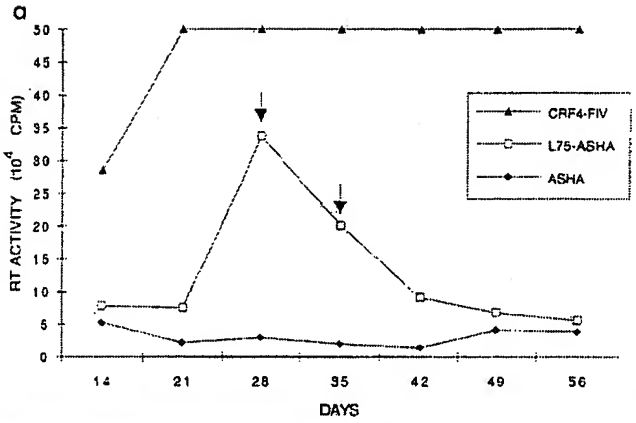


FIG. 1. Seroprevalence of cross-reactive antibodies to feline lentivirus in lions (*P. leo*). (A) Western blot analysis of selected lion serum and plasma samples from free-ranging populations. Lanes 1 to 4, lions Ple-276, Ple-298, Ple-300 (seronegative), and Ple-284, from the Ngorongoro Crater (NGC); lanes 5 to 10, lions Ple-481, Ple-468, Ple-457, Ple-387 (seronegative), Ple-458 (L75), and Ple-556, from the Serengeti ecosystem (SER); lane 11, lion Ple-325, from Lake Manyara (LM); lanes 12 to 15, lions Ple-173, Ple-174, Ple-180, and Ple-164, from Kruger Park (KP); lanes 16 to 19, lions Ple-443, Ple-446, Ple-429, and Ple-431, from Namibia (NAM), all seronegative; lanes 20 to 23, lions Ple-193, Ple-188, Ple-186, and Ple-187, from Asia, all seronegative; lanes + and −, control sera from FIV-positive and FIV-negative domestic cats, respectively. (B) Seroprevalence of a lion lentivirus LLV (FIV-Ple) in African and Asian free-ranging lions. The N value indicates the number of free-ranging animals screened from a given area. Lentivirus seroprevalence percentages are given in numerical and pie graph form. The 55 Indian lions from the Gir Forest include 6 wild-caught lions, 9 animals from the Sakkarbaug Zoo (denoted by the asterisk) in Junagadh that were captured from the Gir Forest sanctuary, and 40 captive-born lions from Sakkarbaug Zoo. All other lions are free-ranging animals. The Masai Mara is an extension, in Kenya, of the Serengeti ecosystem. The Ngorongoro Crater is an extinct volcanic caldera approximately 10 miles in diameter adjacent to the Serengeti. The lion population of approximately 100 animals is descended from 15 founders after a *Stomoxys* epizootic in 1962 (41). Lake Manyara has a small isolated lion population that is well-known because the lions sleep in the trees. The Gir Forest population consists of about 250 lions descended from a near extinction of the Asiatic lion subspecies *P. leo persica* due to overhunting at the turn of the 20th century (32, 53). Namibian lions are from Etosha Pan National Park. (C) Western blot analysis of serum samples from two adult male East African lions. Both lions were sampled in 1987 and in 1989. In lanes 1987, both Ple-517 and Ple-258 lack antibodies to FIV-PET. In lanes 1989, both lions reveal antibodies to the FIV major core protein p26. Ple-517 (PNE) and Ple-258 (Charlie) were both inhabitants of the Serengeti Wildlife Reserve, Tanzania. Lanes + and −, positive (FIV-infected) and seronegative control domestic cat sera, respectively.

lation of lions that reflects a high level of genetic diversity across the study area (32, 38).

The phylogenetic reconstruction of feline lentiviruses from domestic cats (FIV-Fca) and pumas (FIV-Pco), also called puma lentivirus (PLV), revealed species specificity among the cat lentiviruses, with FIV-Pco isolates showing genetic divergences greater than those previously observed between the human lentiviruses and their nearest simian neighbors (26, 36, 44). In this report we summarize a widespread prevalence of exposure to lentiviruses cross-reactive to domestic cat FIV



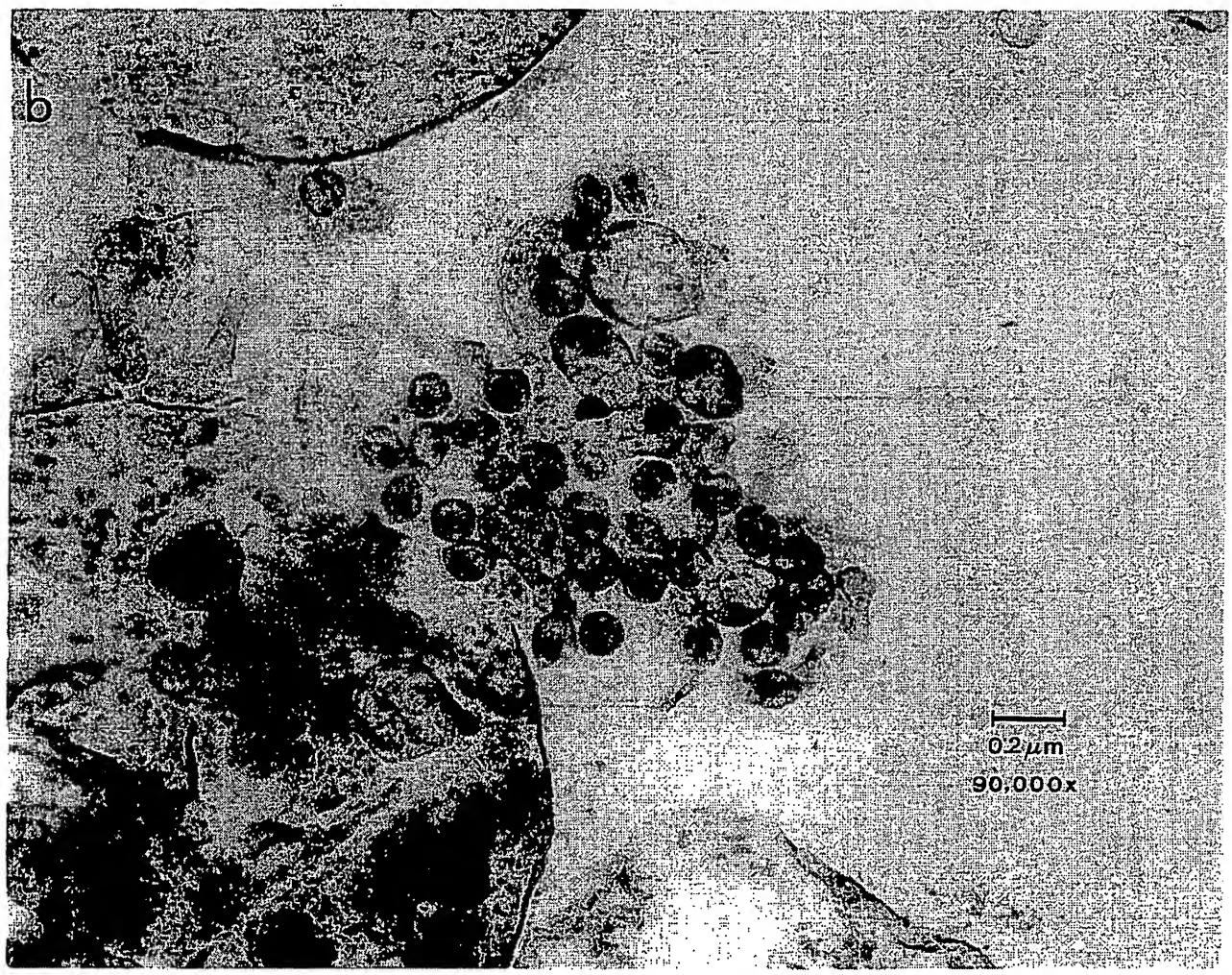
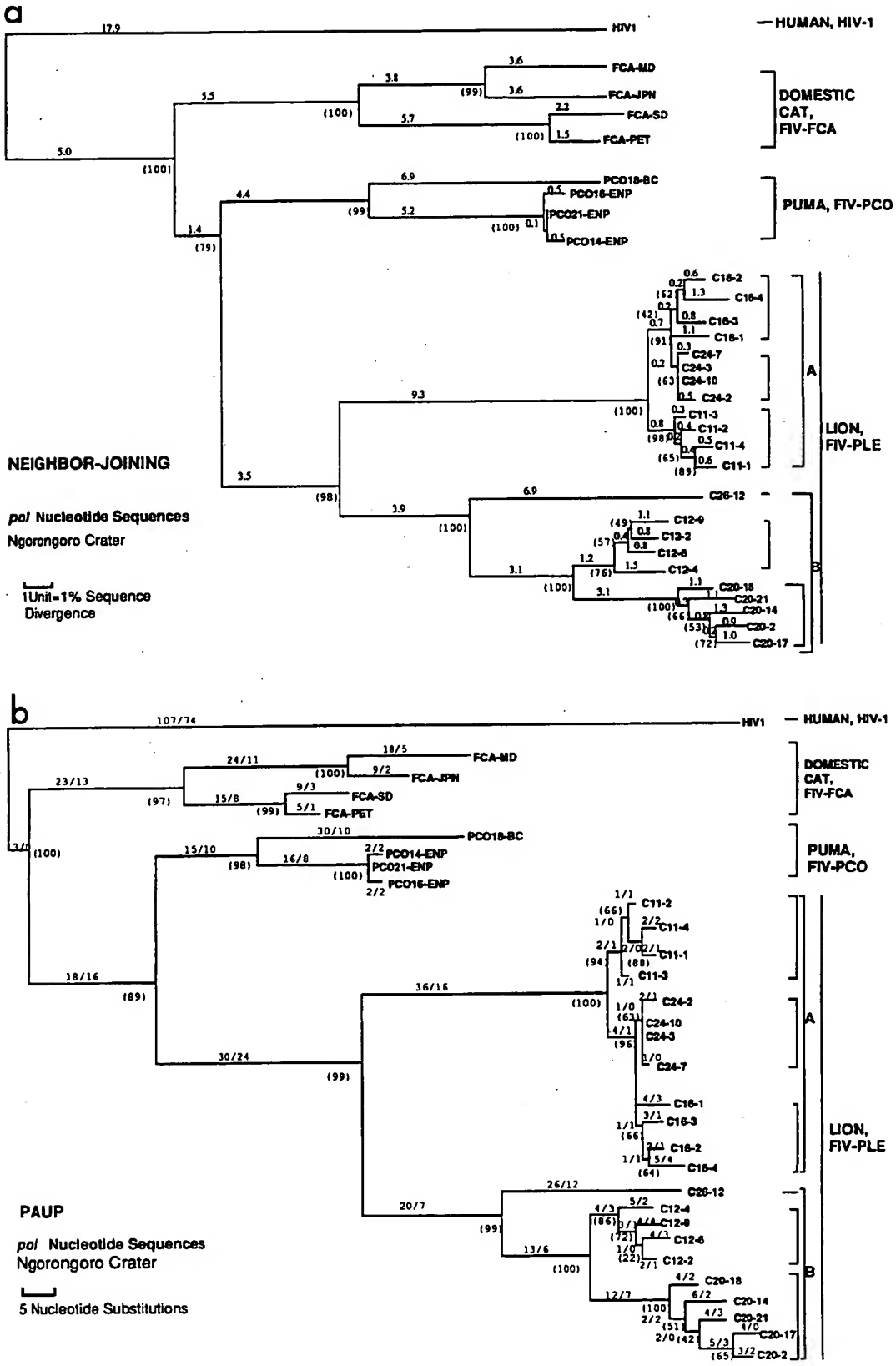


FIG. 2. Transmission of a lion lentivirus (FIV-Ple) to fresh lion PBMCs. (A) Production of magnesium-dependent RT activity for L75 (Ple-458)-Asha (Ple-73) PBMC coculture, Crf4-FIV, an FIV-infected positive control, and Asha PBMCs alone for baseline RT measurements. RT assays containing water in place of culture supernatants were also used for negative controls. The abscissa (x) is defined by days following cocultivation, while the ordinate (y) indicates the RT activity in scintillation counts per minute (CPM). The arrows indicate time points (days 28 and 35) when culture fluids were harvested and processed for electron microscopy. (B) Electron micrograph of mature lentivirus particles in zoo lion PBMCs cocultured with PBMCs from Serengeti lion L75.


a
HIV1
HUMAN, HIV-1
FCA-MD
FCA-JPN
FCA-SD
FCA-PET
DOMESTIC CAT, FIV-FCA
PCO18-BC
PCO16-ENP
PCO21-ENP
PCO14-ENP
PUMA, FIV-PCO
C16-2
C16-4
C16-3
C16-1
C24-7
C24-3
C24-10
C24-2
C11-3
C11-2
C11-4
C11-1
A
LION, FIV-PLE
C26-12
C12-9
C12-2
C12-6
C12-4
C20-18
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B
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pol Nucleotide Sequences
Ngorongoro Crater
1Unit=1% Sequence Divergence
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HIV1
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PCO14-ENP
PCO21-ENP
PCO16-ENP
PUMA, FIV-PCO
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C16-3
C16-2
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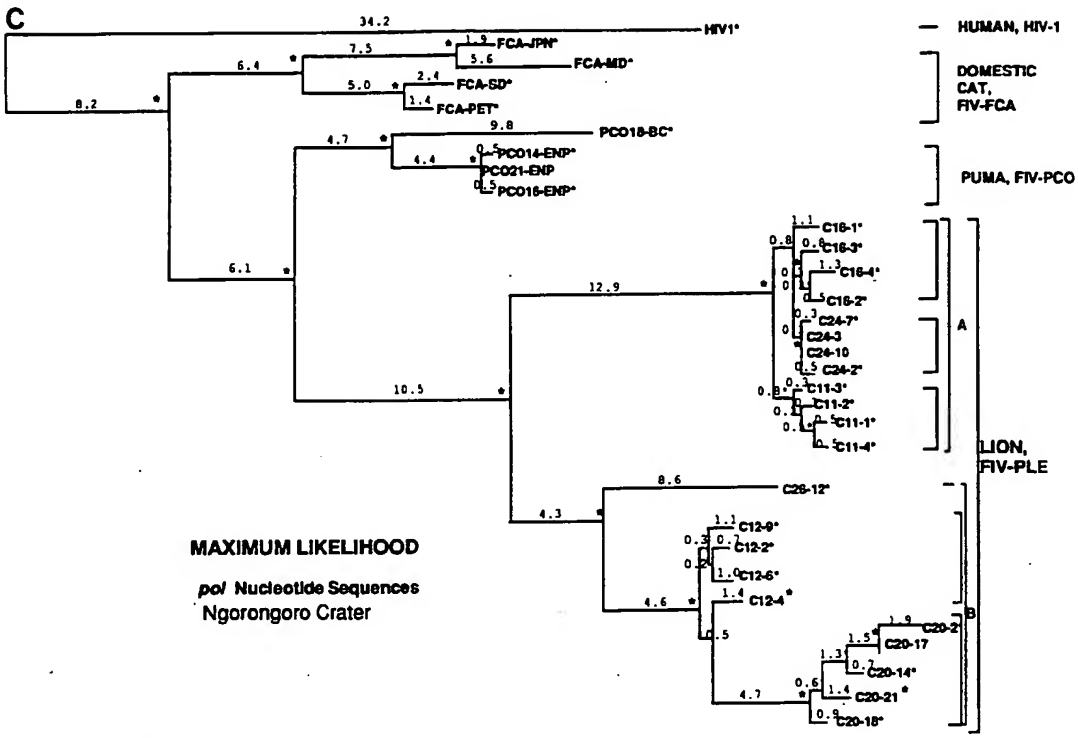
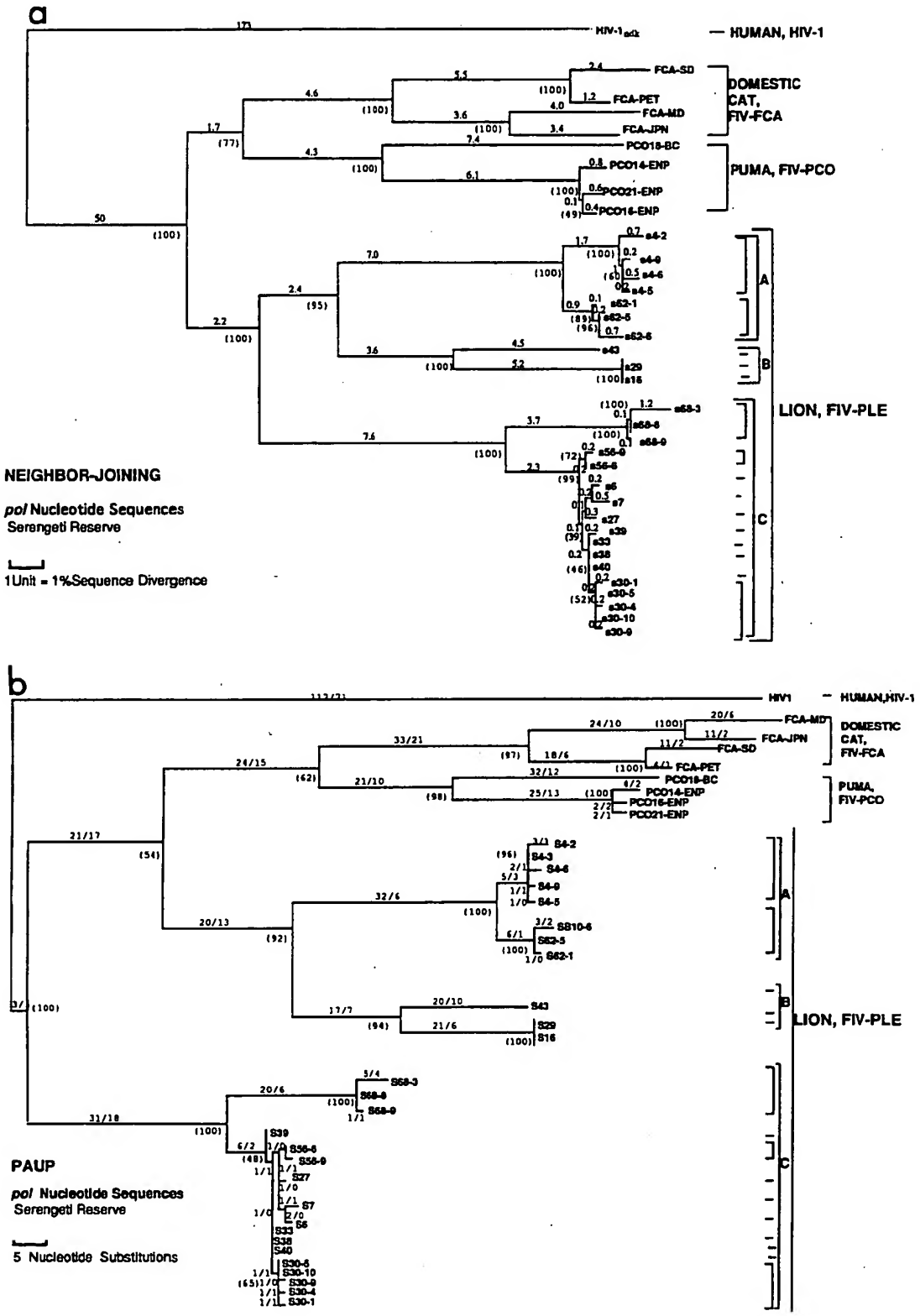


FIG. 3. Evolutionary trees of the 22 Ngorongoro Crater FIV-Ple *pol* sequences from six lions plus homologous FIV cat and puma sequences and HIV *pol* sequences. Each full-length sequence was aligned by using the PILEUP program of the Genetics Computer Group software package (11). Distances are expressed on the basis of percent nucleotide mismatches. (a) Neighbor-joining phenetic tree using a distance matrix based on the algorithm of Saitou and Nei (45) within the PHYLIP (phylogenetic inference package) program, version 3.51c (14). The numbered branch lengths are the percent nucleotide divergence between proviral sequences. In parentheses are the percentages of bootstrap iterations (of 100) that support the adjacent node. Brackets indicate sequences from individual lions and from clade groups. (b) Phylogenetic tree derived from the PAUP (phylogenetic analysis using parsimony) program, version 3.0 (50). A strict bootstrap consensus tree based on a midpoint root and on stepwise addition is shown. The branch lengths are presented as the number of nucleotide substitutions (preceding the shill) along with the number of unambiguous substitutions (following the shill). The number of unambiguous sites is equal to branch length minus the substitutions exhibiting homoplasy. The tree shown has an overall length of 521 changes and a consistency index of 0.70, indicating a 30% convergence level. Bootstrap values (of 100 iterations) are given in parentheses in support of each node. (c) Maximum likelihood tree generated by the DNAML program of the PHYLIP package (14). The final phylogeny of this tree was based on the most likely topology to emerge from the examination of 6,387 trees. The tree was generated by using a transition/transversion ratio of 2. Branch lengths are estimates of substitutions relative to the tree and are shown as 100 times the expected number of substitutions per site. Branch lengths not significantly different from zero were collapsed into polytomies. Asterisks indicate significant support ($P \leq 0.01$) for adjacent divergence node. The ln likelihood for the presented tree is −2,967.80. All trees were rooted with HIV-1 as the outgroup. The number immediately following the virus species abbreviation (e.g., FIV-Ple) represents an animal number, while hyphenated suffixes represent different molecular clones from PCR products of the same lion. The bracketed letter sequence clusters (A, B) denote phylogenetic clades or lineages (see text). The NDK strain of HIV-1 is shown here and was used in all analyses. Virus abbreviations: FIV-Fca, feline immunodeficiency virus (from domestic cat, *Felis catus*); FIV-Pco, feline immunodeficiency virus (from puma, *Puma concolor*); FIV-Ple, feline immunodeficiency virus (from lion, *Panthera leo*). Geographical abbreviations: C, Ngorongoro Crater lions; S, Serengeti reserve; LM, Lake Manyara; KP, Kruger Park; ENP, Everglades National Park pumas; BC, Big Cypress Swamp pumas; MD, Maryland; JPN, Japan; SD, San Diego; PET, Petaluma, CA.

(FIV-Fca) in 409 lions from seven wild populations located in eastern Africa, in southern Africa, and in Asia. Included in sampled populations were lions from the large Serengeti population (n = 3,000 lions), two populations with a history of demographic contraction followed by inbreeding (the Ngorongoro Crater population in Tanzania and that of the Gir Forest Sanctuary in western India), and populations from western and eastern regions of southern Africa (29, 30, 32–34, 41, 53).

To describe the phylogenetic relationship between lentivirus sequences within infected lions, between lions of the same population, and between geographically isolated populations, we determined the sequence of the reverse transcriptase (RT) domain of the slowly evolving *pol* gene from multiple lion lentivirus (FIV-Ple) isolates by using PCR-amplified DNA from frozen leukocytes of representative individuals. A phylogenetic analysis revealed several general trends, namely: (i) monophyly of viral quasispecies within individual lions, supportive of a clonal expansion of infections; (ii) the occurrence of three very deep ancestral clusters (phylogenetic clades) of FIV-Ple which were as different from each other as is domestic cat FIV (FIV-Fca) from puma lentivirus (FIV-Pco) (36); (iii) the occurrence of divergent clades within the same populations but not (so far) in the same individuals; and (iv) the evolutionary association of the three FIV-Ple clades somewhat more recently than the divergence nodes of all lion sequences from other felid lentiviruses (FIV-Fca, FIV-Pco), suggesting that FIV-Ple likely evolved in allopatric (geographically isolated)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HUMAN, HIV-1
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FCA-PET
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FCA-JPN
DOMESTIC CAT, FIV-FCA
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PCO14-ENP
PCO21-ENP
PCO16-ENP
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PCO16-ENP
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DOMESTIC CAT, FIV-FCA
PUMA, FIV-PCO
S4-2
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S62-5
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S33
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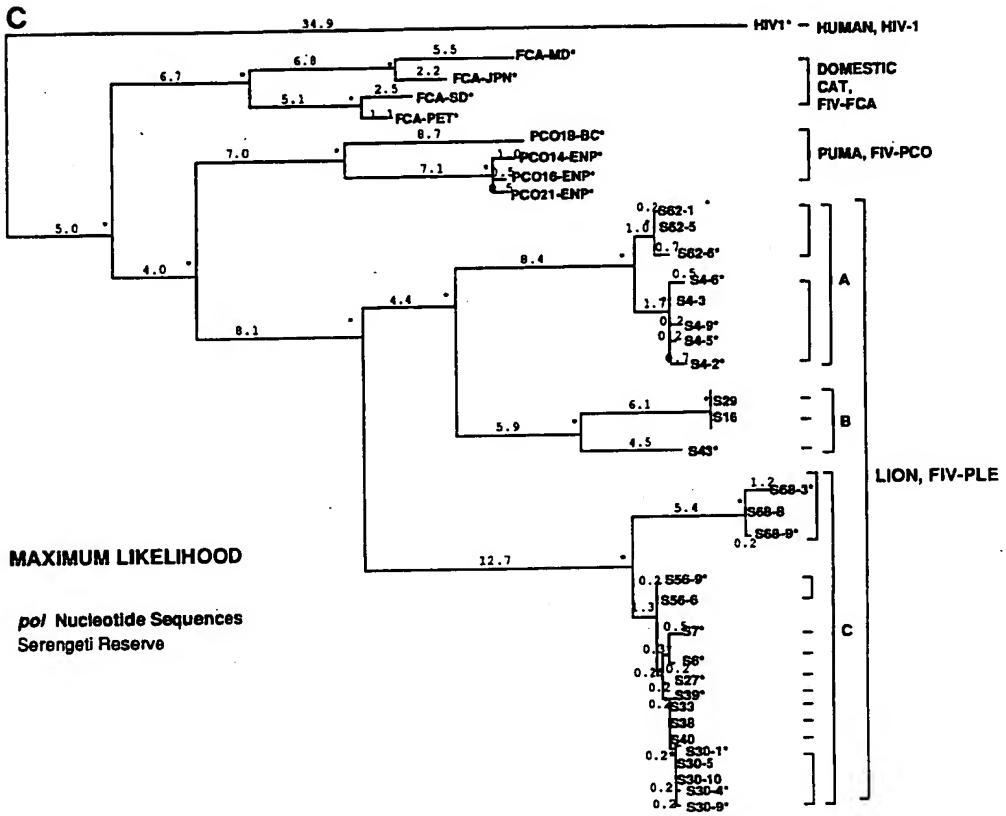


FIG. 4. Evolutionary trees of the 28 Serengeti FIV-Ple sequences from 15 lions and other feline and human lentivirus *pol* genes. Sequences were aligned as described in Materials and Methods. (a) Neighbor-joining phenetic tree using distance matrix as described in the legend to Fig. 3 (45). Branch lengths reflect percent nucleotide divergence. In parentheses are the numbers of bootstrap iterations (of 100) that support the adjacent node. (b) PAUP maximum parsimony tree (50). The strict bootstrap consensus tree presented here has an overall length of 580 changes and a consistency index of 0.65, yielding a 35% convergence level. The scale and branch lengths are presented as the number of nucleotide substitutions (preceding the shill) with the number of unambiguous substitutions (following the shill). An unambiguous site is equivalent to the branch length minus the homoplasy. Bootstrap values (of 100 iterations) are in parentheses. (c) Maximum likelihood tree from DNAML (14). This tree phylogeny was based on the most statistically significant tree of 12,609 trees examined. This tree was generated using a transition/transversion ratio of 2. Branch lengths are substitutions, relative to the tree shown, shown as 100 times the expected number of substitutions per site. Branch lengths not significantly different from zero were collapsed into polytomies. Asterisks indicate significant support ($P \leq 0.01$) for adjacent divergence node. The ln likelihood for the presented tree is −3,217.38. See legend to Fig. 3 for geographic and virus abbreviations.

populations of lions rather than in another felid species, with subsequent and recent transfer to lions. These findings are consistent with the presence of FIV-Ple in some, but not all, lion populations for a long period, possibly as far back as species divergence of the genus *Panthera* (the great cats: lion, tiger, leopard, snow leopard, and jaguar) (52).

(The work reported in this article is in partial fulfillment of requirements for the degree of Master of Science for Eric W. Brown from the Department of Biology, School of Graduate Studies, Hood College, Frederick, Md.)

MATERIALS AND METHODS

Western immunoblot analysis. Serum/plasma and peripheral blood mononuclear cell (PBMC) samples from 409 free-ranging lions were collected from natural populations or wildlife reserves across Africa and Asia from 1983 to 1992 (16, 30, 32, 39, 41). Positive control serum was obtained from experimentally infected cats or seropositive lions, while negative serum came from seronegative cats. Lion serum/plasma samples were tested against FIV-Petaluma domestic cat viral antigens for the presence of cross-reactive antibodies by Western blot as previously described (7, 8, 37).

Samples were collected in full compliance with specific federal fish and wildlife permits (Convention on International Trade of Endangered Species) issued to the National Cancer Institute, principal officer S. J. O'Brien, by the U.S. Fish and Wildlife Service of the Department of the Interior.

Isolation of a lentivirus from lion PBMCs. Heparinized blood was obtained intravenously from free-ranging seropositive Serengeti lions, and the PBMCs were then extracted and purified within 24 h of bleeding by sucrose gradient centrifugation using Histopaque (Sigma). PBMCs from Serengeti lions (10^6 cells) were stimulated by concanavalin A (5 μg/ml) for 72 h and were cultured with mitogen-stimulated donor PBMCs (10^6 cells) from a captive zoo lion that had repeatedly tested negative for virus isolation. Cocultures were propagated in RPMI 1640 with 10% fetal bovine serum and 10% human interleukin-2 (Gibco-BRL). Fresh medium was added every 72 h along with fresh donor cells (10^6) every 14 days. Coculture

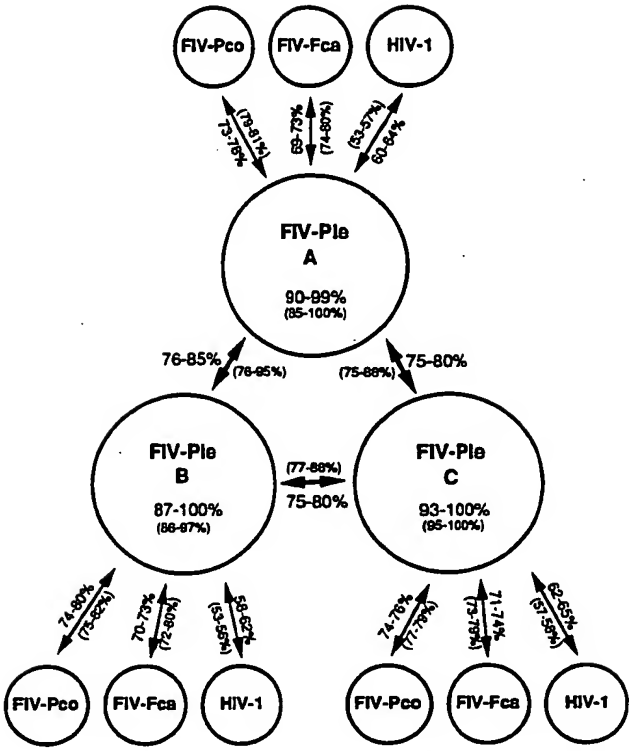


FIG. 5. Schematic showing the minimum and maximum percent nucleotide sequence identities within and between the three major FIV-Ple clades as well as between the FIV-Ple clades and other feline and human lentiviruses. The clade letter designations of the three FIV-Ple clades are present within the large circles (A, B, and C). Minimum-maximum percent sequence identities within each of the three FIV-Ple clades are listed within each clade. Minimum-maximum percent sequence identities between various clades or species are listed along the arrows that designate the viruses being compared. Minimum and maximum percent amino acid similarities are also listed in parentheses within clades, between clades, and between feline and human lentiviruses. Virus abbreviations are as listed in the legend to Fig. 3.

supernatants were clarified by low-speed centrifugation and were examined weekly for the presence of replicating virus by Mg^{2+}-dependent RT assays performed essentially as described previously (6). Infectious supernatant from a Crandall feline kidney monolayer chronically infected with FIV-Petaluma (Crf4) was used as a positive control for RT assays, while supernatant from cultured seronegative lion PBMCs was used to monitor RT baseline activity. During the period of maximal RT activity, culture supernatants were harvested and surveyed by electron microscopy for the presence of lentivirus particles.

Amplification of the proviral *pol* gene from leukocytes of lion lentivirus-infected animals. Genomic DNAs were isolated from the PBMCs of 27 seropositive lions, and DNA concentrations were adjusted to 0.25 μg/μl. The segment of the RT region from the proviral *pol* gene (FIV-Petaluma coordinates bp 2403 to 3042) was amplified by PCR from 0.25 to 0.50 μg of genomic DNA stocks. PCR reactions were set up in 50-μl volumes and were prepared according to conditions outlined in the user's manual of *Taq* DNA polymerase (Perkin-Elmer Cetus) with 1.5 U of *Taq* polymerase per reaction. Thermal cycles were performed in a Perkin-Elmer 9600 under the following conditions: 94°C 3-min burst, followed by 35 cycles each at 94°C (15-s bursts), 45°C (28-s bursts), 72°C (15-s bursts), ending with a 72°C 10-min extension. Seropositive lions and experimentally infected domestic cat PBMC DNAs were used for positive controls, and PBMC DNAs from seronegative lions, negative domestic cats, DNA from other species, and reactions with no templates were used for negative controls. Oligonucleotide primer sequences and the base-pair positions on the FIV-PET (36) genome are 1258F (bp 2430), 5'GAAGCATTAACAGAAATAGTAG3'; 1260R (bp 3007), 5'GTTCTTGTTGTAATTTATCTTC3'; 1259F (bp 2466), 5'GAAGGAAAGGTAAAAAGAGCAGATC3'; 1261R (bp 2990), 5'ATCTTCAGGAGTTTCAAATCCCCA3'; 6635F (bp 2511), 5'CCTATATTTTGCATTAAAAAG3'; 6637R (bp 2944), 5'ACCCCATATGATATCATCC3'; 6636F (bp 2529), 5'AAAGAATCAGGAAAATATA3'; 6638R (bp 2934), 5'GATATCATCCATATATTGATAT3'. Nested primers 1258F, 1260R, 1259F, and 1261R were used to amplify all of the Serengeti FIV-Ples as well as Ngorongoro Crater lion sequences C12, C20, and C26. Nested primers 6635F, 6637R, 6636F, and 6638R were used to amplify C24, C16, C11, LM3, and LM4. Primers 1259F, 1261R, 6635F, and 6637R were used to amplify the Kruger Park sequences. The 1200-numbered primers were designed from the alignment of FIV-Fca and FIV-Pco isolates from Florida panthers (37). The 6600-labeled primers were made from the alignment of Serengeti lion S4 and S62 sequences and represent highly conserved regions of the RT molecule that are shared across lion, puma, and domestic cat FIV sequences.

Molecular cloning and sequence determination. Products from PCR amplification of lion leukocyte DNA were column purified and ligated to a sticky-T cloning vector previously prepared by attaching a single dTTP to the blunt end of a (KS+) phagemid (Stratagene). Products were ligated overnight, and reactions were transformed into an NM522 cloning strain by conventional heat-shock methods (37). Three-milliliter overnight cultures were prepared from 10 clones, and phagemids were then harvested in ion-exchange columns (Qiagen). Multiple phagemid clones were then sequenced by Sanger chain-termination methods on an ABI 373A automated sequencer (Applied Biosystems Inc.). M13 and T3/T7 primer binding sites were used to initiate forward- and reverse-strand reactions. For lions S6, S7, S27, S33, S38, S39, S40, S16, S29, and S43 oligonucleotide primers containing M13 universal primer sequences were used with the first-round PCR products to yield a final product that was then sequenced directly. All sequence data were then transferred into the VAX/GCG (11) sequence handling program, and full-length fragments were assembled.

Phylogenetic reconstruction. Nucleotide sequences from the Serengeti, Tanzania, were subjected to phylogenetic analyses by using a total of 421 bp of nucleotide sequence which, when translated, yielded 139 amino acid residues. Shorter sequences from the Ngorongoro Crater (375 bp, 124 amino acids) and representative sequences (368 bp, 122 amino acids) from the Serengeti, Ngorongoro, Lake Manyara, Tanzania, and Kruger Park, South Africa were also analyzed. Sequences were aligned by the GAP program of the Genetics Computer Group (University of Wisconsin) computer software package (11, 28). Distances are expressed on the outcome of percent nucleotide sequence identity. Gaps were given a weight equivalent to a single nucleotide substitution, regardless of length. Three different phylogenetic algorithms were used. The phenetic algorithm Neighbor-joining, was available through PHYLIP version 3.51c (14, 45). The second method utilizes the principle of maximum parsimony, available in PAUP version 3.0 (50, 51). The third procedure employed the maximum likelihood

approach, available in DNAML in PHYLIP (14). Phenetic (distance matrix based) and maximum parsimony analyses were also performed on the representative FIV-Ple amino acid sequences from all geographic locales.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been deposited into the GenBank sequence database under the following accession numbers: U06010 (llvc11-1), U06011 (llvc11-2), U06012 (llvc11-3), U06013 (llvc11-4), U06014 (llvc12-2), U06015 (llvc12-4), U06016 (llvc12-6), U06017 (llv12-9), U06018 (llvc16-1), U06019 (llvc16-2), U06020 (llvc16-3), U06021 (llvc16-4), U06022 (llvc20-14), U06023 (llvc20-17), U06024 (llvc20-18), U06025 (llvc20-2), U06026 (llvc20-21), U06027 (llvc24-10), U06028 (llvc24-2), U05990 (llvc24-3), U05991 (llvc24-7), U05992 (llvc26-12), U06029 (llvs54-2), U06030 (llvs4-3), U06031 (llvs4-5), U06032 (llvs4-6), U06033 (llvs4-9), U06043 (llvs62-1), U06044 (llvs62-5), U06045 (llvs62-6), U06036 (llvs56-6), U06037 (llvs56-9), U06039 (llvs68-3), U06040 (llvs68-8), U06041 (llvs68-9), U06002 (llvs30-1), U06003 (llvs30-10), U06004 (llvs30-4), U06005 (llvs30-5), U06006 (llvs30-9), U06034 (llvs40), U06035 (llvs43), U06038 (llvs6), U06042 (llvs7), U05999 (llvs16), U06000 (llvs27), U06001 (llvs29), U06007 (llvs33), U06008 (llvs38), U06009 (llvs39), U05997 (llvlm3-9), U05998 (llvlm4-1), U05993 (llvkp153-10), U05994 (llvkp165-8), U05995 (llvkp175-8), and U05996 (llvkp177-9).

RESULTS

Serological incidence of FIV-related lentiviruses in lions. Plasma samples from 406 lions were typed by Western blot for antibodies to feline lentiviruses antigenically related to FIV (Fig. 1a). The serological status for FIV cross-reacting antibodies of free-ranging African and Asian lions from seven populations is presented in Fig. 1b. As previously reported (7, 8, 37), exposure to FIV-related lentiviruses appears to be endemic in East African lion populations. The incidence of seropositive lions was 84% in the Serengeti, 70% in the Ngorongoro Crater, 80% in Lake Manyara, and 91% in Kruger Park. Two lion populations, Asiatic lions from India and African lions from Etosha Pan in Namibia, were all seronegative (Fig. 1a and b) (8, 37).

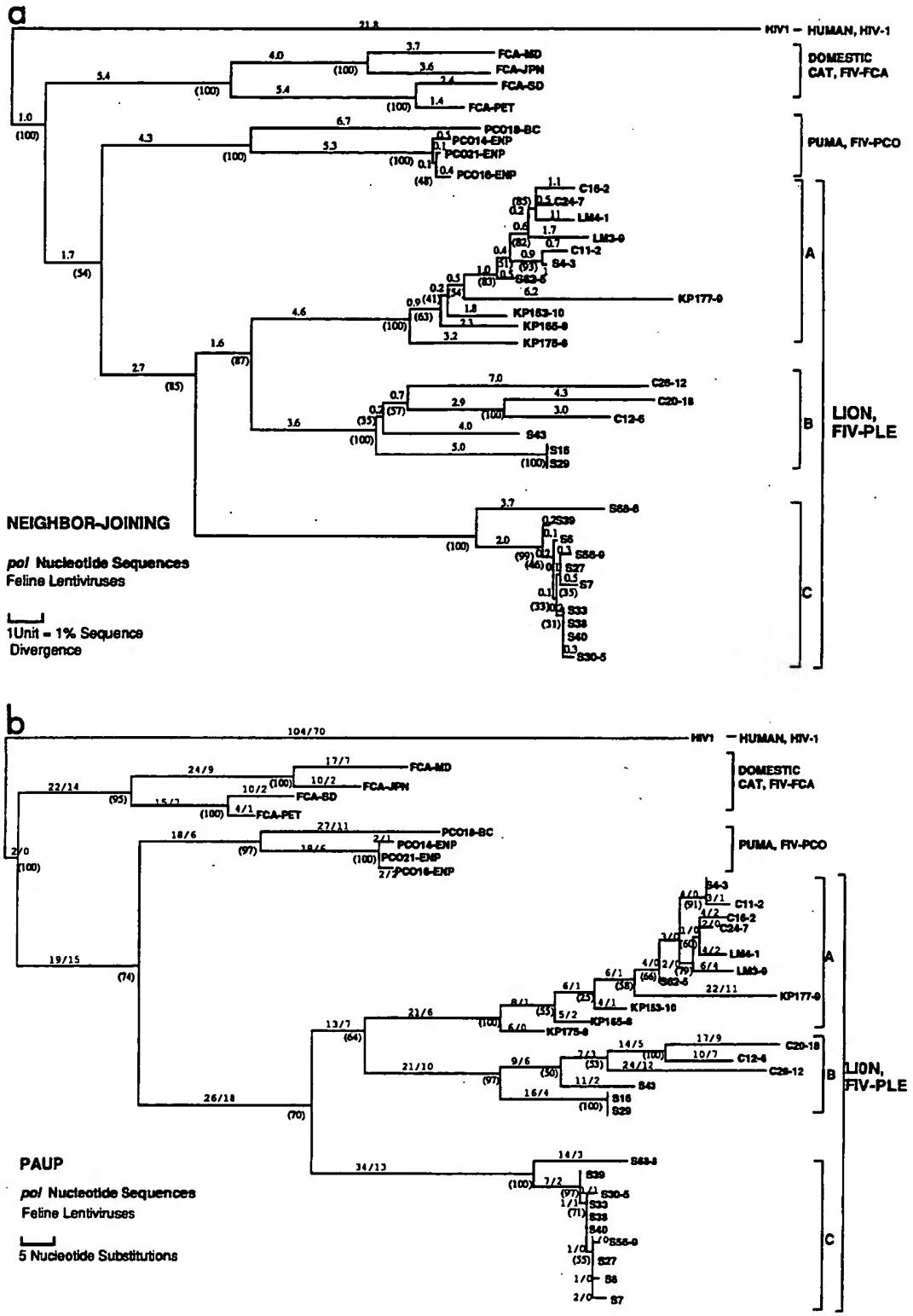
Natural seroconversion (transition from antibody-negative to -positive) in two East African male lions was observed (Fig. 1c). Multiple serum samples were drawn from these two individuals in different years. At the first sampling date in 1987, both animals were seronegative, but by 1989 each animal possessed antibodies to the major FIV core protein (p26). These findings affirm that, like domestic cat FIV (54), a lion lentivirus (FIV-Ple) is being transmitted horizontally within the species, possibly through bites during fighting. A comparison of FIV antibody status in parents with that in cubs was possible, as we had previously established the parentage of 72 cubs by using feline-specific DNA fingerprints (16, 39). Eighteen percent (20 of 113) of cubs born to seropositive dams tested seronegative, and 20% (7 of 30) of cubs produced by two seropositive parents also were antibody negative. Five separate litters born to infected mothers were documented as having at least one seronegative cub, while two of these litters ($n = 3$ cubs per litter) were entirely free of FIV-Ple exposure. Conversely, 77% (7 of 9) cubs born to seronegative dams tested seropositive. The parent-cub discordance would suggest that horizontal transmission and not maternal infection is the primary route of transfer between free-ranging lions.

Isolation of lentivirus particles from lion PBMCs. PBMCs from five seropositive Serengeti lions were cocultured with donor PBMCs from Asha (Ple-73), a captive lion from The National Zoological Park, Washington, D.C. The supernatant from one lion, L75 (Ple-458), resulted in elevated magnesium-dependent RT activity (338,000 cpm) at 28 days (Fig. 2a), indicating that a replication-competent retrovirus was transferred to the fresh PBMCs. A second coculture of fresh L75 lymphocytes was repeated; it also tested positive for RT activity. At the peak of RT activity, electron microscopic examination of the L75-Asha coculture supernatants (Fig. 2b) revealed the presence of individual virus particles with lentivirus morphology, including a bar-shaped cylindrical core characteristic of the primate lentiviruses. These lion lentivirus particles likely originated from L75, since Asha lymphocytes, although antibody positive for FIV p26, remained consistently RT and virus isolation negative when cultured with lymphocytes from two different seronegative lions and alone. Virus isolation from other lions was unsuccessful (Fig. 2a).

Phylogenetic characterization of FIV-Ple from Ngorongoro Crater lions. The nucleotide sequence of a 375-bp amino-terminal segment of the FIV-Ple *pol* gene was first determined from molecular clones of PCR-amplified products from lymphocyte DNA samples of six infected Ngorongoro Crater lions. The aligned nucleotide sequences of the Ngorongoro FIV-Ples were compared with FIV-Pco (PLV), FIV, and HIV-1.

The Ngorongoro Crater FIV-Ple sequences were analyzed by using three different phylogenetic approaches: phenetic or distance matrix based, maximum parsimony, and maximum likelihood. The derived phylogenetic trees revealed several important aspects (Fig. 3). First, in almost all cases the trees were topologically equivalent, irrespective of the phylogenetic method employed. The only exceptions involved the relative positions of multiple clones from a single lion. Second, in every case, clones from a single lion exhibited monophyly with respect to their host; that is, each clone sequence had another clone from the same lion as its nearest relative. Third, two very divergent phylogenetic lineages or clades, designated clades A and B, were resolved. The two clades appear among lions in the same small population. Lions C12 and C26 were inhabitants of the same pride, as were C24 and C11, with the two FIV-Ple clades spanning four prides overall in the Ngorongoro Crater. Fourth, the extent of sequence divergence between the two FIV-Ple clades (20 to 24% for nucleotide sequences and 10 to 18% for amino acid sequences) was large, on the order of the genetic distances between homologous *pol* regions observed for highly divergent geographic isolates of puma lentivirus, FIV-Pco (maximum 25% nucleotide sequence divergence; Fig. 3a) (37); the divergence was twice that previously reported for FIV (FIV-Fca) *pol* sequence variation (maximum 16% nucleotide divergence and 11% amino acid sequence divergence). FIV-Ple clade divergence exceeds the divergence in the homologous *pol* region between HIV-2 and SIV-SM (17% nucleotide sequence divergence and 10% amino acid divergence) and in the homologous *pol* region between HIV-1 and SIV-CPZ (19% nucleotide divergence and 6% amino acid sequence divergence); further, the extreme divergence in FIV-Ple clades approaches the *pol* sequence difference observed between HIV-1 and HIV-2 (30% nucleotide difference and 28% amino acid difference).

Phylogenetic characterization of FIV-Ple from Serengeti lions. The nucleotide sequences from 421-bp segments of the *pol* gene from 15 seropositive lions collected from the large outbred Serengeti population were collected and aligned for phylogenetic analysis. The Serengeti FIV-Ple sequence data were analyzed by the same three phylogenetic methods (Fig. 4). Multiple cloned sequences were taken from the following Serengeti lions: S4, S62, S56, S30, and S68. As was seen with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FCA-SD
FCA-PET
PUMA, FIV-PCO
PCO18-BC
PCO14-ENP
PCO21-ENP
PCO16-ENP
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C16-2
C24-7
LM4-1
LM3-9
C11-2
S4-3
S62-5
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C20-18
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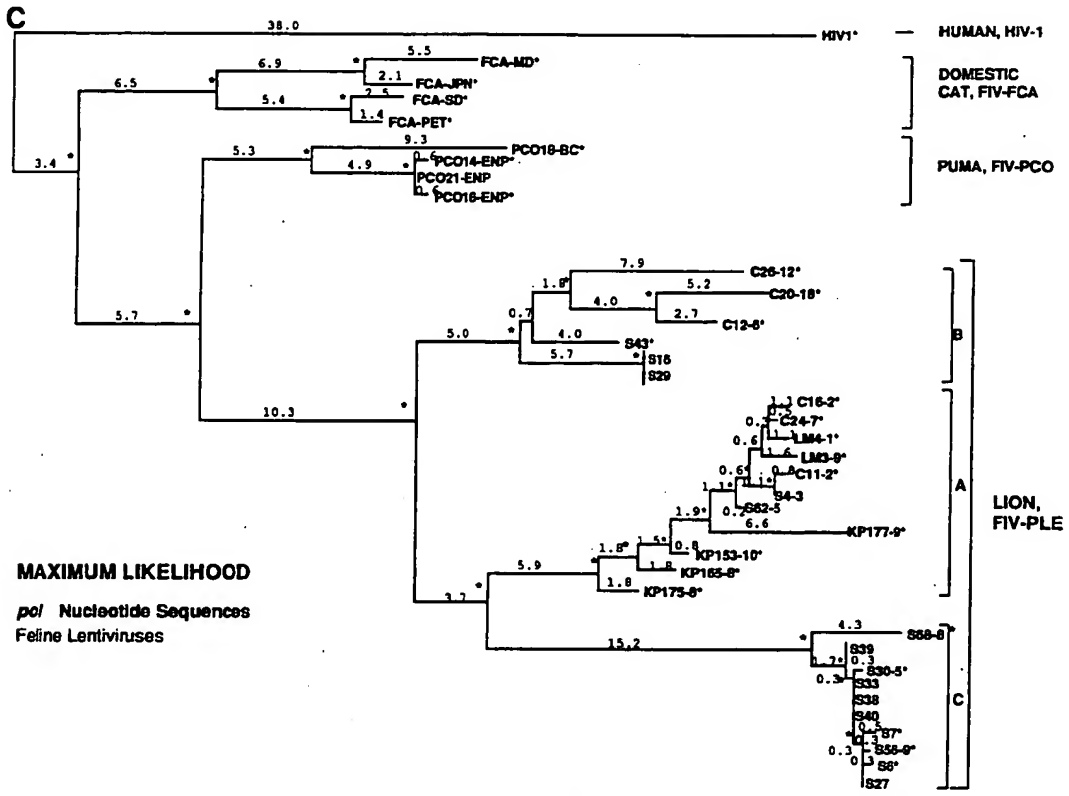


FIG. 6. Phylogenetic trees of the 27 representative African FIV-Ple nucleotide sequences along with other feline and human lentivirus *pol* sequences. (a) Neighbor-joining tree based on the distance matrix described in the legend to Fig. 3 (45). Branch lengths reflect the percent nucleotide divergence. In parentheses are the number of bootstrap replications (of 100) in support of the adjacent node. (b) PAUP maximum parsimony tree. This strict bootstrap consensus, based on a midpoint root and on stepwise addition, has an overall branch length of 634 substitutions and a consistency index of 0.60, indicating a 40% convergence level. Branch lengths are expressed as the number of nucleotide substitutions along with the number of unambiguous substitutions (branch length minus homoplasy). Bootstrap values (of 100 iterations) are presented. (c) Maximum likelihood tree. This topology was determined from 11,360 trees examined. This tree was generated by using a transition/transversion ratio of 2. Branch lengths are estimates of substitutions and are shown as 100 times the expected number of substitutions per site. Branch lengths not significantly different from zero are collapsed into polytomies. Asterisks indicate significant support ($P \leq 0.01$) for adjacent divergence node. The ln likelihood for the presented tree is −3,467.66. All trees were rooted with HIV-1 as an outgroup. See legend to Fig. 3 for a complete description of the methods used and for virus and geographic abbreviations.

samples from lions from the Ngorongoro Crater, all of the cloned segments from within the same individual are monophyletic, suggesting clonal expansion of the infecting virus followed by the evolution of quasispecies. The two divergent lion clades, A and B, observed with lions from the Ngorongoro Crater were also evident for the Serengeti lions. However, a third sequence clade, designated clade C, also appeared. Clade C sequences show a greater divergence from those of either clade A or clade B than exists between clade A sequences and clade B sequences. Further, the nucleotide divergence (26%) between clade C sequences and those of the other two lion lentivirus (FIV-Ple) clades appears to be greater than any distances previously described for domestic cat lentivirus (FIV-Fca) or puma lentivirus (FIV-Pco) isolates (36, 37).

Phylogenetic characterization of FIV-Ple from all geographic locales. We have analyzed a single representative FIV-Ple sequence from each of 27 lions examined from throughout their African range, along with FIV-Fca, FIV-Pco, and HIV-1. Sequences from 6 Ngorongoro lions, 15 Serengeti lions, 2 Lake Manyara lions, and 4 Kruger Park lions were included in the analysis. The minimum and maximum percent nucleotide sequence identities between the three FIV-Ple clades are shown in Fig. 5 along with the percent sequence identity between each lion clade and FIV-Fca, FIV-Pco, and HIV-1.

Phylogenetic analysis of the FIV-Ple sequences using three phylogenetic methods led to the following observations (Fig. 6). First, the three FIV-Ple clades were found throughout the range of African lions. Bootstrap support for the three clades was very strong (100% for each with Neighbor-joining and 97 to 100% for maximum parsimony (Fig. 6a and b), as was seen in Serengeti (Fig. 4a and b) and Ngorongoro Crater (Fig. 3a and b) FIV-Ple phylogenetic analyses. The topology of the trees was consistent for all methods applied, with the exception of the maximum-likelihood algorithm, which placed clade B as the ancestral clade among the three. Clade A FIV-Ple viruses were present in three contiguous East African lion populations of the Serengeti, Ngorongoro, and Lake Manyara as well as in lions from Kruger Park, South Africa. Clade B FIV-Ple sequences were found in the Ngorongoro Crater and the Serengeti, while clade C was unique to the Serengeti FIV-Ples. Second, the nodes adjoining the three FIV-Ple clades appear to be very deep, displaying a unique trichotomy of sequence divergence. Third, the maximum divergence observed between

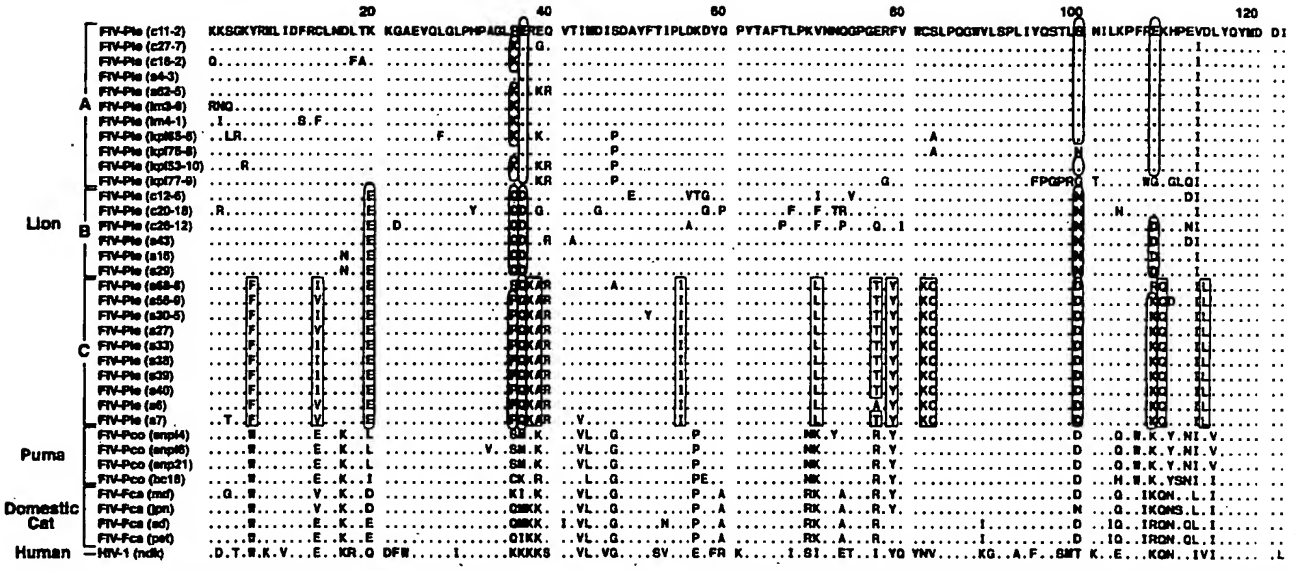


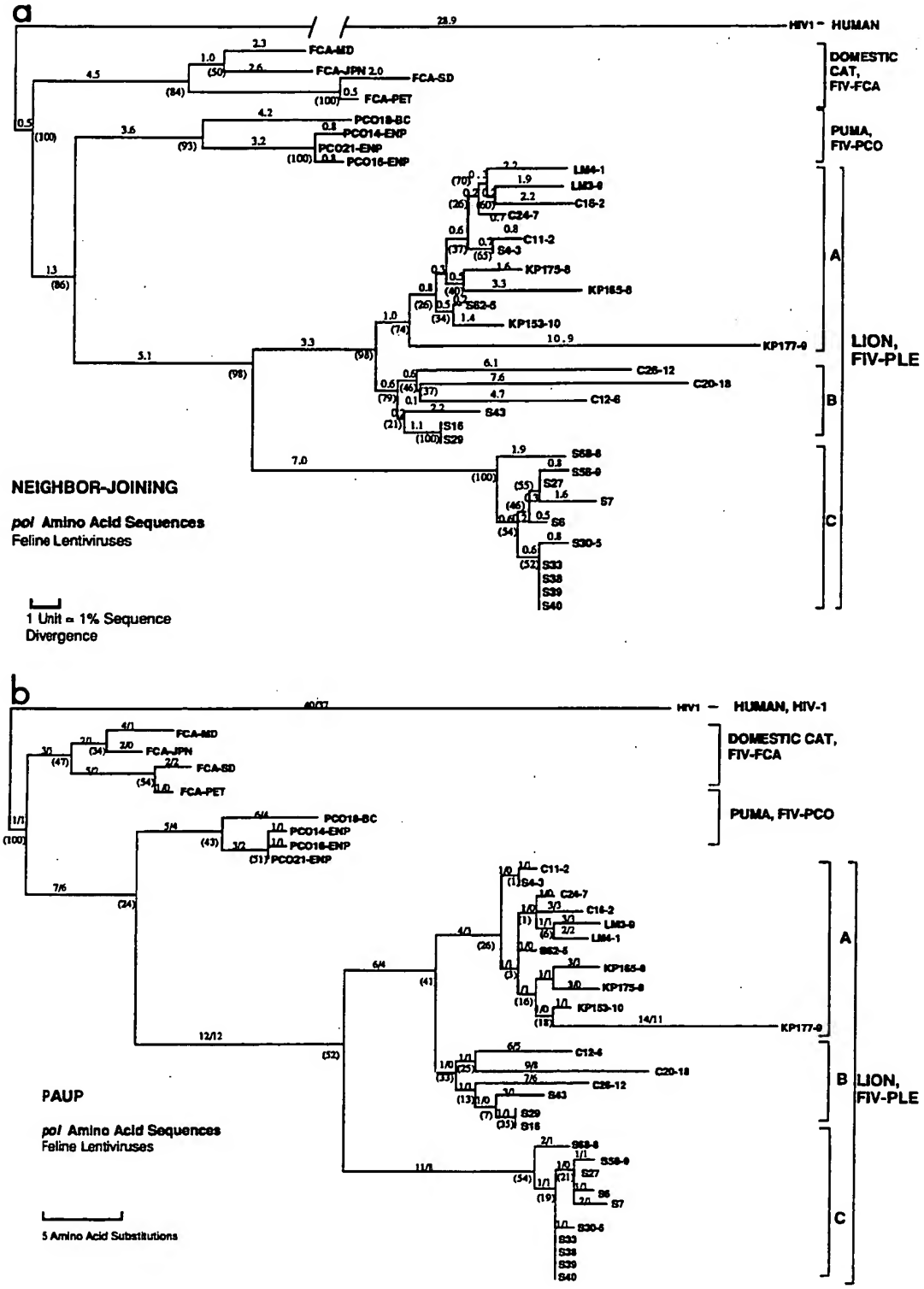
FIG. 7. Alignment of the predicted amino acid sequences of a conserved RT domain in the FIV-Ple, FIV-Pco, FIV-Fca, and HIV-1 *pol* genes (11, 28). Dots below the reference sequence indicate identical amino acids. FIV-Ple lineages are denoted A, B, and C at the left along with labels indicating puma, domestic cat, and human. The alignment contains representative amino acid sequences from FIV-Ple sequences from all geographic locales and from other feline and nonfeline lentiviruses. The shaded ovals indicate the shared derived characters, signature synapomorphies, that are unique to each of the three FIV-Ple clades. The open oval indicates the synapomorphic residue the distinguishes clades B and C from clade A, while the open rectangles denote those residues that distinguish clade C from the other two clades. See legend to Fig. 3 for virus and geographic abbreviations.

the three FIV-Ple clades surpasses the genetic distances between domestic cat FIVs and puma FIV-Pcos (Fig. 5 and 6a). Finally, the FIV-Ple sequence clades appear to be monophyletic with respect to lions, such that any FIV-Ple sequence clade has as its nearest neighbor another FIV-Ple sequence clade.

Analysis of FIV-Ple *pol* amino acid sequences from all locales. The FIV-Ple nucleic acids were translated by computer (11), and the resultant amino acid sequences of representative FIV-Ples from all geographic locales have been analyzed. The aligned amino acid sequences of this data set are presented in Fig. 7. Immediately, it became apparent that there are very clear regions across the RT domain in which variation is permitted. Conversely, there appear to be regions that do not change and that may be functionally constrained. These conserved regions demonstrate homology across all feline lentiviruses including HIV-1 (Fig. 7). Further, there appear to be shared residues present in the FIV-Ple amino acid alignment that are unique to a given FIV-Ple clade. These shared derived characters, or signature synapomorphies, are present in several positions across the RT region. Synapomorphic residues at positions 36-37, 100, and 108 are unique in each of the three FIV-Ple clades, while residues 6, 14, 20, 38 to 39, 55, 70, 77, 79, 82-83, and 114 distinguish clade C from the other two groups (Fig. 7). Furthermore, a single synapomorphy at residue 20 distinguishes clades B and C from clade A (Fig. 7).

The phylogenetic reconstruction of the *pol* amino acid sequences was derived with the Neighbor-joining and PAUP algorithms (45, 50). The evolutionary trees derived from these analyses are presented for the total FIV-Ple datum set in Fig. 8. The *pol*-based amino acid trees recapitulate the topologies of the major FIV-Ple lineages, A, B, and C, which were described with the nucleotide trees. However, the topology of several of the branch tips of the amino acid trees varies from that observed in the nucleotide analysis, possibly demonstrating the divergence plateau that these sequences may have reached due to the functional selection constraints of the RT molecule (4, 27). Also, the nodes separating the three FIV-Ple groups did not exhibit the depth of clade divergence observed in the nucleic acid trees. This observation held true for the domestic cat and puma lentiviruses as well. Consistent with the nucleotide sequence analysis, these amino acid trees of the RT domain also reveal unusually large genetic diversity among African lions and monophyly with respect to host species.

FIG. 8. Neighbor-joining (45) phenetic and PAUP (50) maximum parsimony trees of the RT amino acid sequences from representative FIV-Ples and other feline lentiviruses. Sequences were aligned by the PILEUP program (11) which used the algorithm of Needleman and Wunsch (28). Gaps are given a weight of one residue difference (37). (a) Neighbor-joining tree using distance matrix of amino acid sequences from FIV-Ples from all geographic locales. Branch lengths indicate percent amino acid divergence between the translates. Negative branch lengths were allowed. Bootstrap values (of 100 iterations) are indicated in parentheses in support of each node. (b) PAUP maximum parsimony tree based on a strict bootstrap consensus and on a midpoint root with the branch-swapping and steepest descent options in effect. The tree shown has an overall length of 194 changes and a consistency index of 0.84, indicating a 16% convergence level. The scale and branch lengths are presented as the number of amino acid substitutions (preceding the shill), along with the number of unambiguous sites (synapomorphies) (following the shill). The number of unambiguous sites is equal to branch length minus homoplasy. Bootstrap values (of 100 iterations) are given in parentheses for the respective nodes. See legend to Fig. 3 for abbreviations and for a description of the methods used.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HIV1 – HUMAN
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PUMA, FIV-PCO
LION, FIV-PLE
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FCA-JPN
FCA-SD
FCA-PET
PCO18-BC
PCO14-ENP
PCO21-ENP
PCO16-ENP
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LM3-8
C16-2
C24-7
C11-2
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Feline Lentiviruses
5 Amino Acid Substitutions

DISCUSSION

This study demonstrates the seroprevalence and phylogenetic divergences of FIV-related lentiviruses in several free-ranging populations of lions. The seroprevalence of FIV in lions exceeds the incidence of cross-reactive antibodies to FIV in all other species of cat (7, 8, 37) and to SIV in free-ranging primates (2, 24, 35). Interestingly, an intercontinental difference was observed with respect to lentivirus exposure. The absence of seroprevalence in Asian lions is reminiscent of the absence of SIV antibodies in free-ranging Asian primates (10, 19). In Africa, lions in Namibia, West Africa, appear free of infection, while East African lions are infected at significant levels. This finding can be interpreted in two ways. FIV-Ple infection may not have emerged in lions until subsequent geographic partitioning of the species or, alternatively, FIV-Ple infection may have been present since the radiation of the lion species but may have failed to persist due to a founder effect of seronegative ancestors of West African lions.

We have presented evidence documenting two cases of seroconversion in male Serengeti lions. These data are reminiscent of the horizontal transmission of infection with FIV in domestic cats through biting (54, 55). Frequent horizontal transmissions of this nature, combined with the highly social and aggressive nature of lions (38, 40), may help to account for the high rates of exposure in the lion populations. The epidemiological data regarding the FIV status of cubs revealed no significant trends supporting the transfer of FIV-Ple infection from mothers to cubs. A large percentage (77%) of positive cubs was born to seronegative dams, while 18% of the cubs born to infected dams remained free of antibodies at the time of sampling. Although we cannot exclude the possibility that some dams may have seroconverted following giving birth but before our sampling dates, these data suggest that maternal transmission (in utero or via milk) is not the sole mechanism of transfer for the virus and probably is not the major route of transmission to other lions.

The phylogenetic analysis of multiple sequences from the same lion has revealed patterns of variation that are consistent with the formation of viral quasispecies in vivo (13, 20). Various levels of sequence divergence within infected lions have been observed, with ranges of from less than 1% to more than 4% at the nucleotide level. Fluctuations in the amount of diversity within an infected lion may reflect the length of time an animal has been infected with the virus. In all cases in which multiple clones were derived from a single animal, the sequences were monophyletic with respect to the individual host. These findings suggest a single point of infection followed by the generation of genetic variants in situ (13, 20, 22, 31).

The phylogenetic analysis of the FIV-Ple *pol* gene revealed the occurrence of three distinct clades with large sequence divergence levels compared with clades of previously described feline lentiviruses (37, 44). The genetic distance between FIV-Ple clade sequences exceeds that between homologous *pol* sequences found in comparisons of other cat species and is on the order of the distances between lentiviruses from different species of cats. The most primitive clade appears to be clade C, which was found only in the Serengeti, although not all analyses implicated clade C (e.g., Fig. 6c). The deepness of the clade divergences suggests a rather ancient separation of the clades, either in distinct cat species recently introduced into lions or in geographically isolated (allopatric) lion populations that have converged rather recently. The former explanation would gain credence if a lentivirus closely related to one of the clades were discovered in sympatric feline species (e.g., leopards, domestic cats, cheetahs, or African wild cats). Alternatively, the apparent monophyly of the three lion clades within the lion species would support their origin within allopatric lion populations. Whichever the final explanation, the lion lentivirus divergence is likely quite ancient, perhaps extending back to the radiation of the genus *Panthera* into the great cats—lion, leopard, jaguar, etc.—estimated at 1.6 to 2.0 million years ago (47, 52).

It is not yet clear whether the occurrence of FIV-related lentiviruses is associated with immune deficiency or with any pathology in exotic cat species. Although there is compelling evidence for FIV-mediated CD4 T-lymphocyte depletion and associated loss in immune response in domestic cats (1, 9), this has not been observed in wild feline species. However, neither are there sufficient data to conclude that FIV-Ple infection is benign. A thorough analysis of T-cell subsets, immune response, and potential pathology will be required before any firm conclusions can be drawn. If FIV-Ple proves to have little pathology, the situation would be reminiscent of the endemic SIV infection, with no clinical symptoms, in several free-ranging African monkey species. In contrast, Asian macaques manifest an AIDS-like illness when infected with SIV but do not appear to be exposed to SIV in their natural habitat. It seems as if the African species have reached a host-virus accommodation or symbiosis over time, because of either natural selection of genetically resistant host survivors, natural genetic attenuation of viral pathology, or a combination of both. The examples of endemic lentivirus infection in lions and other wild felids provide a rare opportunity to investigate naturally selected solutions to historic outbreaks of debilitating viral infections.

ACKNOWLEDGMENTS

We are very grateful to A. R. Hoelzel, R. Benveniste, and D. Derse for useful discussion; to Stanley Cevario, Walter Knott, and Janice Martenson for excellent technical assistance; to M. Gonda and K. Nagashima for electron microscopy; and to the staff and management of the Frederick Biomedical Supercomputing Center, National Cancer Institute, for allotting required computer time used in the sequence and phylogenetic analyses.

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Efficacy evaluation of prime–boost protocol: canarypoxvirus-based feline immunodeficiency virus (FIV) vaccine and inactivated FIV-infected cell vaccine against heterologous FIV challenge in cats

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Objective: To evaluate the immunogenicity and prophylactic efficacy of immunization schemes employing a recombinant canarypoxvirus ('ALVAC')-based feline immunodeficiency virus (FIV) vaccine alone or in combination with an inactivated FIV-infected cell vaccine against homologous and heterologous FIV challenges in cats.

Methods: Specific pathogen-free cats were given a total of three immunizations with subtype A vaccines and challenged 4 weeks after the final immunization with 50 median animal infectious doses (ID_{50}) of FIV-Petaluma, a subtype A isolate. Following the initial challenge, protected cats received a second challenge with 75 ID_{50} of FIV-Bangston, a subtype B isolate. FIV-specific humoral and cell-mediated responses were measured to determine the immune correlates of protection.

Results: Two of three cats immunized with the ALVAC–FIV recombinants alone were protected from homologous FIV challenge in the presence of FIV-specific cytotoxic T-lymphocyte (CTL) responses but in the absence of FIV-specific humoral responses. All three cats immunized with the ALVAC–FIV recombinant and boosted with FIV-infected cell vaccine were also protected from homologous FIV challenge in the presence of both FIV-specific CTL and humoral responses. Partial to full protection was observed in ALVAC–FIV/FIV-infected cell vaccine-immunized cats against a heterologous FIV challenge given 8 months after the initial challenge. Two out of three cats had transient infection and the remaining cat had no sign of FIV infection at a dose at which all three control cats were readily infected.

Conclusions: Immunization schemes employing ALVAC-based FIV vaccines in combination with inactivated FIV-infected cell vaccine generate protective immune responses that can cross-react with FIV isolates that are genetically distinct from the vaccine strains.

AIDS 1998, **12**:11–18

Keywords: Feline immunodeficiency virus, vaccine, canarypoxvirus-based recombinant vaccine, cytotoxic T-lymphocyte, virus-neutralising antibodies, T-helper

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Sponsorship: This work was supported by a NIH/NIAID grant (RO1-AI30904) and a Virogenetics Corporation/University of Florida collaborative grant.

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Date of receipt: 26 March 1997; revised: 28 August 1997; accepted: 9 September 1997.

Introduction

Based on genetic variation in the *env* and *gag* coding regions, HIV isolates obtained world-wide have been classified into several subtypes or clades [1,2]. Optimally, a vaccine against HIV should induce immune responses that can cross-react with a wide variety of these subtypes. Thus far, HIV vaccine trials have concentrated mainly on a single subtype, subtype B, because this represents the predominant type found in Europe and the United States. However, the emergence of HIV isolates other than subtype B is increasing. Furthermore, 90% of the reported HIV cases are in developing countries where the HIV epidemic encompasses multiple subtypes. These countries in particular would benefit from the development of multiple subtype vaccines because drug treatments, even if these become available, would be too expensive.

The infection of cats with the feline immunodeficiency virus (FIV) provides a good model to assess the efficacy of vaccine strategies against multiple subtypes. FIV is a natural pathogen in cats and causes an immunodeficiency syndrome that closely resembles AIDS in humans [3,4]. Like HIV, FIV strains have been classified into four subtypes (A–D) based on genetic differences predominantly in the envelope (Env) amino-acid sequence and, to a lesser extent, in the Gag amino-acid sequence [5–7]. Vaccine protection against homologous and slightly heterologous FIV strains (within one subtype) has been achieved with inactivated whole virus and inactivated (FIV-infected) cell vaccines (ICV) [8–11]. Unlike the results from simian immunodeficiency virus (SIV) rhesus studies [12,13], such protection was not mediated by allogeneic major histocompatibility complex (MHC) antigens present in the vaccine, because cats immunized with uninfected cells used to generate ICV were not protected against the same challenge [8]. However, these same vaccines failed to induce protective immunity against distinctly heterologous FIV strains of other subtypes [10,11,14]. Thus, a modified or different vaccine approach is required to induce immune responses that will cross-react with a wide variety of FIV subtypes. One approach carried out with some success in the chimpanzee model has been the use of vaccines composed of whole Env or Env fragments from multiple isolates [15]. These vaccines were shown to confer protection against the vaccine strains as well as against an HIV strain that differed by approximately 18% in the Env amino acids from the vaccine strains. An alternative to the use of vaccines composed of antigenic determinants from multiple isolates may be the use of viral vector-based vaccines. These vaccines differ from conventional vaccines, such as those discussed above, in that they are more effective in priming cell-mediated responses. The epitopes recognized by cell-mediated responses may be directed against epitopes that are more conserved among different isolates and as such provide protection against a wider variety of HIV isolates.

One viral vector, evaluated in recent trials of HIV vaccines, is the canarypoxvirus-derived vector ALVAC [16,17]. ALVAC-based vaccines are considered safe due to their restricted host range and their inability to undergo full replication in cells of non-avian origin [17]. The efficacy of ALVAC-based vaccines has been tested successfully against a number of viral pathogens including lentiviruses [16–23]. Macaques immunized with ALVAC recombinants expressing Env and Gag epitopes of HIV-2 and boosted with recombinant Env were protected from homologous HIV-2 challenge [20]. Additionally, ALVAC–HIV-1 vaccines have proved effective against homologous HIV-1 challenge in a small number of chimpanzees [22,23]. More significantly, macaques immunized with an ALVAC recombinant vaccine encoding epitopes of HIV-1 were shown to be partially protected from heterologous challenge with HIV-2, which shares only 40–60% homology with HIV-1 [21]. Although the numbers of animals in these trials were small, these findings suggest that immunization schemes involving ALVAC-based vaccines may be able to afford protective immunity against a wider variety of strains and subtypes when compared with immunization schemes involving conventional protein-based vaccines.

The aim of this study was to determine whether immunization protocols involving ALVAC-based FIV vaccines in combination with ICV can induce protective immunity against homologous and heterologous FIV challenge in cats. In this pilot study, cats were immunized with ALVAC-based FIV vaccines expressing the Env and Gag epitopes of FIV and boosted with ICV. Protection was evaluated initially against challenge with a homologous subtype A isolate, FIV-Petaluma (FIV_{Pet}), and subsequently in protected cats against challenge with a heterologous subtype B isolate, FIV-Bangston (FIV_{Bang}).

Materials and methods

Animals

Specific pathogen-free (SPF) cats, aged 14 weeks, were purchased from Liberty Research, Inc. (Waverly, New York, USA), and housed and cared for in accordance with policies set by the Environmental Health and Safety of the University of Florida.

Vaccine preparation

Canarypoxvirus (ALVAC)–FIV recombinants were generated using strategies similar to those described previously [18]. Briefly, the coding regions of

$FIV_{Ville\ Franche}$ (subtype A) *env*, *gag* and protease genes were amplified by polymerase chain reaction (PCR) and placed under the transcriptional control of early/late vaccinia promotors. The promotor–gene constructs were then cloned into a donor plasmid (pC6L) and inserted into the non-essential C6 region of ALVAC by homologous recombination. The ALVAC–*env/gag/pol* recombinant (hereafter referred to as ALVAC-FIV) was amplified and titrated onto permissive chicken embryo fibroblasts. The ALVAC-FIV vaccine and ALVAC vector control were prepared from a serum-free lysate of infected chicken embryo fibroblasts. ALVAC immunizations were given at 1×10^8 plaque-forming units intramuscularly. ICV consisted of 2×10^8 paraformaldehyde-inactivated FL-4 cells (a feline lymphoid cell line chronically infected with FIV_{Pet}) mixed with 250 µg SAF-MDP (Syntex Adjuvant Formulation–muramyldipeptide) adjuvant and was given subcutaneously.

Grouping and immunization protocol

The first challenge study involved nine cats divided into three groups. The first group of three cats (QA6, QH3, PY4) received two immunizations with ALVAC-FIV followed by one boost with ICV; the second group of three cats (QS4, QC3, PY3) received three immunizations with ALVAC-FIV. The control group of three cats (QG4, QC5, QE4) received two immunization with the parental ALVAC vector alone followed by one booster immunization with ICV. Previous studies in our laboratory have demonstrated that a single immunization with ICV failed to induce protective immunity against low dose homologous and heterologous FIV challenge (unpublished observation). All immunizations were given at monthly intervals. The second challenge study involved six cats, the ALVAC-FIV/ICV-immunized group (PY4, QH3, QA6), and a control group of three age-matched SPF cats (EJ2, DH3, GU5), which had received no immunizations prior to the FIV_{Bang} challenge.

Challenge inoculum

The initial challenge inoculum consisted of 50 median animal infectious doses (ID_{50}) of cell-free FIV_{Pet} [subtype A, primary peripheral blood mononuclear cells (PBMC)-derived] and was given intraperitoneally 4 weeks after the final immunization. The second challenge inoculum consisted of 75 ID_{50} cell-free FIV_{Bang} (subtype B, primary PBMC-derived) and was given intraperitoneally 8 months after the initial challenge.

Immunogenicity monitoring

The induction of FIV-specific antibody responses was determined by immunoblotting (Western blot) [8]. The titre of FIV-specific antibodies was determined by 10-fold serial dilutions of serum samples (1 : 100 to $1 : 1 \times 10^6$) and presented as the reciprocal of the highest dilution (in $\log_{10}$) at which FIV-specific bands could be visualized. Similarly, antibody responses to ALVAC proteins were determined by immunoblot analysis using clarified lysates of permissive chicken embryo fibroblasts infected with ALVAC vector. Viral neutralizing antibody (VNA) responses were determined on PBMC using previously described assays [8]. The presence of FIV-specific cytotoxic T-lymphocyte (CTL) responses was measured in peripheral blood by methods similar to those described by Song *et al.* [24] using autologous FIV-infected PBMC as target cells. The induction of FIV-specific T-helper proliferative responses was measured in peripheral blood upon exposure to inactivated FIV by standard ^{3}H-thymidine incorporation assays [8].

Viral infectivity monitoring

Viral infection was monitored by several methods. This included assessment of viral reverse transcriptase (RT) activity in PBMC, bone-marrow and lymph-node cells taken at various times post-challenge by previously described methods [8]. In addition, proviral DNA (latent infection) was monitored by PCR using FIV Env-specific primers on DNA extracted from PBMC, bone-marrow and lymph-node cells upon culturing for RT activity [8]. Furthermore, FIV infection was determined by monitoring and comparing the profile of FIV-specific humoral responses and VNA responses in serum taken before and after challenge.

In vivo assessment of viral status

Four SPF cats (RU1, RU2, RU3, DE4) were given a total of 1×10^7 cells obtained 20 weeks after the FIV_{Bang} challenge from ALVAC-FIV/ICV-immunized cats (QA6, PY4, QH3) or an FIV_{Bang}-infected ALVAC control cat (DH3). Cells consisted of 3×10^6 PBMC, 7×10^6 bone-marrow cells, and 1×10^6 lymph-node cells. Prior to transfer, cells were washed in sterile phosphate-buffered saline (PBS) and resuspended in 2 ml PBS. After cell transfer, all cats were monitored for viral status using methods described above.

Results

Homologous FIV_{Pet} challenge

In the initial study, the prophylactic efficacy of ALVAC-based FIV vaccines alone or in combination with ICV was evaluated against experimental infection with 50 ID_{50} of FIV_{Pet} (subtype A). This FIV isolate was identical to the isolate used to prepare ICV and differed only slightly (3% in the Env and 1% in the Gag amino-acid sequence) from the $FIV_{Ville\ Franche}$ isolate (subtype A) used to generate the ALVAC-FIV recombinant vaccine.

Following challenge, all three control cats became viremic as determined by virus isolation (RT) for infectious virus and by FIV-specific PCR for proviral DNA

in peripheral blood and tissue samples (bone-marrow and lymph-node cells; Table 1). Furthermore, these cats developed high titres of VNA responses (> 100 VNA titre), indicative of active infection. In contrast, all three cats immunized with ALVAC-FIV and boosted with ICV, and two out of three cats immunized with ALVAC-FIV alone remained free of virus as determined by RT and PCR on PBMC, lymph-node and bone-marrow tissues tested up to 8 months post-challenge (Table 1). Interestingly, ALVAC-FIV/ICV-immunized cats had low titres of VNA responses (5–20 VNA titre) 3 months after challenge (Table 1). These titres, however, were significantly lower than those detected in infected control cats (> 100 VNA titre). The lower titre of VNA responses may suggest that these responses were induced as an anamnestic response to the FIV challenge inoculum or as a low level response to the limited transient infection, and were not caused by active viral infection. This was further supported by the fact that, at 8 months post-challenge, VNA titres of cats QA6 and QH3 dropped to undetectable levels, whereas the VNA titre of cat PY4 remained low (5–20 VNA titre; Table 2). In the case of active infection, an increase rather than a decrease in VNA titres would be expected [8,10].

To determine the immune correlates of protection, humoral and cell-mediated responses were monitored in selected cats from each group after immunization and before challenge. FIV-specific humoral responses determined by immunoblotting were detected only in those cats that received a booster immunization with ICV and were absent in cats that were immunized with ALVAC-FIV recombinant alone (Table 1). These immunoblot results were similar to our results from an enzyme-linked immunosorbent assay using FIV transmembrane peptide substrate with a sequence common to both FIV_{Pet} and FIV_{Bang} [10] (data not shown). However, all cats immunized with ALVAC alone or ALVAC-FIV developed high titres of antibodies to ALVAC, indicating successful vector expression (data not shown). VNA titres were undetectable in all cats prior to challenge even in those boosted with ICV.

Viral vector-based vaccines are in general considered to be effective at eliciting cell-mediated responses. As one measure of cell-mediated immunity, PBMC from a selected number of cats were tested for FIV-specific CTL activity. Freshly isolated PBMC from immunized cats were cultured in the presence of FIV antigen-specific cells and assayed for their ability to lyse autologous PBMC infected with FIV_{Pet}. FIV-specific CTL responses were detected in both the ALVAC-FIV and ALVAC-FIV/ICV-immunized groups. In contrast, the ALVAC/ICV control group had no significant levels (<6%) of FIV-specific CTL activity. As an additional measure for cell-mediated immunity, selected cats in each group were tested for proliferation response of lymphocyte to inactivated FIV_{Pet}. Following immunization, low levels of FIV-specific proliferative responses were detected in one cat from the ALVAC-FIV-immunized group and another one from ALVAC-FIV/ICV-immunized group (Table 1, post-challenge T-helper cell response).

Heterologous FIV_{Bang} challenge

The protected ALVAC-FIV/ICV-immunized cats (QA6, QH3, PY4) were next challenged with a distinctly heterologous FIV isolate from a different subtype. The second challenge consisted of 75 ID_{50} of cell-free FIV_{Bang} (PBMC-derived) and was given 8 months

Table 1. Immune parameters and viral status after immunization and FIV_{Pet} challenge.

			Post-immunization immune status				Post-challenge immune status			Post-challenge viral status (RT/PCR)‖			
Cat	Vaccine (2×)	Boost (1×)	WB titre*	VNA titre	CTL (%)†	T-helper (SI)‡	WB titre*§	VNA titre§	T-helper (SI)‡§	PBMC	Lymph node	Bone marrow	Protection rate*
QA6	ALVAC-FIV	ICV	+ (5–6)	< 5	27.3	NT	+ (4–5)	5–20	NT	–/–	–/–	–/–	
QH3	ALVAC-FIV	ICV	+ (5)	< 5	55.2	NT	+ (3–4)	5–20	NT	–/–	–/–	–/–	3/3
PY4	ALVAC-FIV	ICV	+ (5)	< 5	8.0	2.2	+ (4–5)	5–20	1.3	–/–	–/–	–/–	
QG4	ALVAC	ICV	+ (4–5)	< 5	5	1.0	+ (5)	100	1.2	+/+	+/+	–/–	
QC5	ALVAC	ICV	+ (3–4)	< 5	NT	1.3	+ (5–6)	> 100	NT	–/+	+/+	–/+	0/3
QE4	ALVAC	ICV	+ (3–4)	< 5	NT	NT	+ (5–6)	> 100	NT	+/+	NT	+/+	
QS4	ALVAC-FIV	ALVAC-FIV	– (<2)	< 5	7	NT	+ (5–6)	> 100	1.8	+/+	+/+	–/+	
QC3	ALVAC-FIV	ALVAC-FIV	– (<2)	NT	18.3	2.5	– (< 2)	NT	NT	–/–	–/–	–/–	2/3
PY3	ALVAC-FIV	ALVAC-FIV	– (<2)	< 5	36.7	1.5	– (< 2)	< 5	NT	–/–	–/–	–/–	

*FIV-specific antibody titres by Western blot (WB), defined as maximum dilution (in $\log_{10}$) at which antibodies could be detected. The FIV-specific antibody titres derived from FIV_{Pet} immunoblot strips were identical to those obtained from FIV_{Bang} immunoblot strips. †Cytotoxic T-lymphocyte (CTL) responses given at effector to target cell ratios of 20–25 : 1. ‡T-helper cell activity was determined by T-cell proliferative responses to FIV presented as a mean stimulation index (SI) of triplicate samples. SI >2 was considered to be significant. §FIV antibody titres (WB) determined 2 months post-challenge, VNA responses determined 2–3 months post-challenge, and T-helper cell responses determined 1 month post-challenge. ‖Virus isolation (RT) and polymerase chain reaction (PCR) were performed on peripheral blood mononuclear cell (PBMC) samples collected at 3–4 week intervals for 24 weeks and at 8 months post-challenge and on lymph-node and bone-marrow cells collected at 20 weeks post-challenge and 8 months post-challenge. Negative results represent no detection of virus at any of the timepoints tested. Positive results represent virus detection at multiple timepoints. NT, Not tested.

Table 2. Immune parameters and viral status before and after the FIV_{Bang} challenge.

	Pre-second challenge*			Post-second challenge†										
		VNA titre		VNA titre			RT/PCR on PBMC (weeks post-challenge)							*In vivo* virus detection‡
Cat	WB titre	FIV_{Pet}	FIV_{Bang}	FIV_{Pet}	FIV_{Bang}	WB titre	2	6	10	14	18	22	26	(20-week tissues)
QA6	+ (4–5)	< 5	< 5	5–20	< 5	+ (3–4)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–
QH3	+ (4)	< 5	< 5	5–20	< 5	+ (3)	–/–	–/–	–/–	–/+	–/+	–/–	–/–	–
PY4	+ (4–5)	5–20	5–20	5–20	< 5	+ (3–4)	–/–	–/–	–/–	+§/+	–/–	–/–	–/–	–
GU5	0 (< 2)	< 5	< 5	NT	< 5	+ (4–5)	–/–	+/+	+/+	+/+	+/+	NT	NT	NT
DH3	0 (< 2)	< 5	< 5	NT	>100	+ (5)	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+
EJ2	0 (< 2)	NT	< 5	NT	< 5	+ (4)	–/–	+/+	+/+	+/+	+/+	NT	NT	NT

*Serum samples taken 8 months after FIV_{Pet} challenge, on the day of FIV_{Bang} challenge. †Serum samples taken 4 months after FIV_{Bang} challenge. ‡As an additional method for testing viral status, tissues [peripheral blood mononuclear cells (PBMC), lymph-node, and bone-marrow cells] taken 20 weeks after FIV_{Bang} challenge were inoculated into naive specific pathogen-free cats and the tissue-inoculated cats were monitored for viral status by virus isolation, polymerase chain reaction (PCR), and immunoblot analysis [Western blot (WB)] throughout 24 weeks of study. §Weakly positive by reverse transcriptase (RT; 2 × background level). NT, Not tested, animal euthanized; VNA, viral neutralizing antibody.

after the initial challenge without any intervening booster. In this study, ALVAC-FIV/ICV group with only heterologous challenge was not available. Therefore, the heterologous FIV_{Bang} challenge dose was increased to 75 ID_{50} as a means of overcoming the potential boosting effect of homologous challenge. FIV_{Bang} belongs to subtype B and differs from FIV_{Pet} (subtype A) by 21% in Env and 2.4% in Gag amino-acid sequence. Three age-matched SPF cats (EJ2, GU5, DH3) served as controls for the FIV_{Bang} challenge.

All control cats became viremic at 6 weeks post-challenge as determined by RT, PCR, and immunoblot analysis. FIV_{Bang}-specific neutralizing antibody responses were detected in one of the infected control cats (Table 2). This was consistent with previous studies in our laboratory in which only certain cats infected with FIV_{Bang} developed VNA responses, whereas for unknown reasons others failed to develop these responses [25]. Of the ALVAC-FIV/ICV-immunized group, one cat (QA6) remained virus-negative by all criteria throughout the study period. Another cat (PY4) in this group tested positive for virus by PCR and had low levels of RT titres at 14 weeks post-challenge, but remained virus-negative thereafter. Similarly, cat QH3 tested positive for virus by PCR only at 14 and 18 weeks post-challenge but remained virus-negative thereafter. Nucleotide sequence analysis of the PCR products from these cats confirmed the presence of only FIV_{Bang}-specific sequences (data not shown).

Prior to the FIV_{Bang} challenge, FIV-specific antibody responses were present in all immunized cats (Table 2, Western blot titre). Control cats lacked such responses before challenge as expected, but developed these responses by 8 weeks following the FIV_{Bang} challenge. VNA responses were absent in all cats at the time of challenge except for cat PY4, which had low VNA titres to both FIV_{Pet} and FIV_{Bang}. Following FIV_{Bang} challenge, low VNA titres were detected in all three ALVAC-FIV/ICV-immunized cats. However, these responses were specific for FIV_{Pet} and did not cross-react with FIV_{Bang}.

As an additional means of analyzing the viral status in these cats and to detect low-grade active infection, PBMC, bone-marrow and lymph-node cells taken 20 weeks after the FIV_{Bang} challenge were inoculated into naive SPF cats. All three cats that received cells from ALVAC-FIV/ICV-immunized cats remained virus-negative throughout the 24 weeks of study, whereas the control cat that received cells from a FIV_{Bang}-infected control cat became viremic (Table 2).

Discussion

In this study, cats immunized with a recombinant ALVAC-FIV vaccine and boosted with ICV were completely protected from experimental infection with the homologous FIV_{Pet} isolate, whereas those immunized with the ALVAC-FIV alone were partially protected. Prior to challenge, FIV-specific proliferative responses were detected in one cat from the ALVAC-FIV-immunized group and another from the ALVAC-FIV/ICV-immunized group. These observations were consistent with those of ALVAC-based HIV vaccine candidates in macaques and chimpanzees [20–23]. In non-human primate trials, humoral responses specific for the inserted antigens were weak or undetectable unless a subunit protein booster was given. In addition, the induction of VNA required boosting with HIV Env or peptides corresponding to the V3 region. Similarly, Phase 1 clinical trials in human volunteers assessing the immunogenicity of prime–boost protocols with ALVAC-HIV-1 and subunit protein boost demonstrated that boosting with Env and Gag proteins

significantly enhanced immunogenicity, as exhibited by the induction of detectable antibody responses including VNA [26–28]. In a recent study, chimpanzees immunized with ALVAC–HIV-1 recombinants without protein boosts developed low levels of specific antibody responses including VNA [23]. However, these responses could only be detected after a minimum of four immunizations.

The induction of humoral responses after a single ICV boost is consistent with previous studies in our laboratory in which ICV vaccines were found to elicit FIV-specific humoral responses but failed to induce VNA responses after a single immunization (unpublished observation). The 10-fold higher titre of FIV-specific antibodies in cats primed with ALVAC-FIV may indicate the presence of FIV-specific T-helper responses resulting in a more efficient generation of FIV-specific humoral responses upon exposure to ICV. In fact, low levels of FIV-specific proliferative responses were detected in some of the cats immunized with ALVAC-FIV recombinant alone. The level of these responses were low in comparison with those induced upon multiple inoculations with ICV-based vaccines (stimulation index, 4–6), indicating the inefficiency of recombinant ALVAC-based FIV vaccines in priming such responses [10]. Priming of low levels of T-helper responses has also been observed in chimpanzees immunized with ALVAC recombinants encoding the HIV *env* gene, and in human volunteers immunized with various ALVAC–HIV vaccine candidates (unpublished data).

The ALVAC-FIV vaccine induced significant levels of FIV-specific CTL responses without the need for protein boosts, supporting the findings from other ALVAC vaccine trials. These CTL responses were found to be MHC-restricted since no specific lysis could be detected when non-autologous FIV-infected PBMC were used as target cells (data not shown). However, our study did not distinguish between CTL responses restricted by MHC class I from those restricted by MHC class II. Immunization with canarypoxvirus-vectored vaccines in other species have been reported to elicit CD8+ and CD4+ CTL responses that are MHC class I- and II-restricted, respectively [26–29]. Thus, future studies using purified CD8+ and CD4+ effector populations against infected target cells with selective MHC expression (i.e., skin cell target for MHC class I expression and B-cell target for MHC class I and II expression) may help decipher the nature of the MHC restriction as well as the effector cell phenotype. In our study, FIV-specific CTL responses were detected after a single immunization. This is in contrast to ICV, which when combined with certain adjuvants were able to induce CTL responses but required a minimum of three to four immunizations before such responses were detected [30–32]. The efficiency of ALVAC-based vaccines in eliciting CTL responses may be attributed to the preferential expression of endogenously produced antigens in the context of MHC.

Despite the presence of humoral and cell-mediated responses prior to challenge, it is not clear what constituted protective immunity in the protected cats. It is unlikely that FIV-specific antibody responses alone were responsible for the observed protection. This view is supported by our finding that ALVAC-FIV-immunized cats were protected in the absence of such responses at the time of challenge and that ALVAC/ICV control cats became infected despite the presence of these responses. Moreover, protection did not correlate with the presence of VNA responses as no VNA were detected in the protected ALVAC-FIV- and ALVAC-FIV/ICV-immunized cats prior to challenge. The low titre VNA responses detected in ALVAC-FIV/ICV-immunized cats shortly after challenge, however, may have an important role. This phenomenon has also been observed in macaques immunized with ALVAC–HIV-2 recombinants [20]. Immunized macaques developed VNA titres upon HIV-2 challenge in the absence of clear viremia. Whether these responses are crucial to protection remains to be determined.

The vaccine protection observed in our study may be attributed to CTL responses, as both ALVAC-FIV and the ALVAC-FIV/ICV cats developed FIV-specific CTL responses, whereas the controls cats lacked such responses. However, this view is contradicted by our finding that cat QS4 became infected despite a high level of FIV-specific CTL responses. Nevertheless, these findings suggest the possibility that a combination of both humoral and cell-mediated responses are necessary for vaccine protection. Thus, the partial protection observed in cats immunized with ALVAC-FIV alone may be due to lack of antibody responses, whereas the lack of protection in the ALVAC/ICV control cats may be due to the lack of CTL responses and to the low titre of FIV-specific antibody responses. The need for a combination of immune responses has also been proposed by others with inactivated whole virus vaccines [30]. In these studies, protection against homologous FIV challenge correlated with the presence of both Env-specific CTL and FIV-specific humoral responses. Furthermore, they postulated that humoral responses including VNA responses are required for initial clearance of the virus from the circulation and that virus-specific CTL responses are required to clear virus from reservoir organs such as the lymphoid tissues.

Following the initial challenge, the ALVAC-FIV/ICV-immunized cats had partial to full protection against a second challenge with a heterologous subtype isolate. Immunized cats, compared with control cats, had a

delay in infection and were mostly negative for virus isolation (RT), suggesting a transient infection or at least a significant reduction in viral load. Prior to the FIV_{Bang} challenge, FIV-specific antibody responses were present in all immunized cats and absent in control cats. In addition, cat PY4 had low VNA titres that cross-reacted with FIV_{Bang}. However, this cat was the first of the ALVAC-FIV/ICV-immunized cats to become transiently positive by PCR analysis and had a single transient titre of RT response. Thus, the presence of FIV_{Bang}-specific VNA antibodies prior to challenge is not indicative of full protection. Furthermore, a role for non-neutralizing FIV-specific antibody responses cannot be excluded. For example, resistance to FIV superinfection correlated with high titres of FIV-specific humoral responses, including high VNA titres to only initial virus prior to second virus challenge [25]. Similarly, chimpanzees immunized with recombinant Env and V3 peptides corresponding to two HIV-1 isolates were protected from heterologous challenge in the presence of high titres of HIV-specific antibody responses, including VNA that neutralized the vaccine strains but not the challenge strain [15]. In contrast, monkeys infected with an attenuated macrophage-tropic SIV strain were resistant to superinfection with a highly virulent SIV strain differing more than 16% in the Env amino-acid coding region [31]. In these studies, the length of infection and the concomitant broadening of humoral immune responses positively correlated with protection. On the basis of these studies, it can still be speculated that FIV-specific antibodies present prior to the FIV_{Bang} challenge may have an important role in the removal or reduction of viral load in the immunized cats.

In addition to humoral responses, CTL responses may be, in part, responsible for the partial to full protection observed in our study. The presence of FIV_{Pet}-specific CTL activity was detected in these cats. Unfortunately, cross-reactivity to FIV_{Bang} was not evaluated due to limited amounts of PBMC that could be harvested from these cats. However, the generation of cross-reactive CTL responses against FIV_{Bang} has been detected in cats that were long-term infected with the FIV_{Pet} isolate (unpublished data). Therefore, it is possible that the ALVAC-FIV/ICV immunizations similarly induced cell-mediated responses directed against epitopes conserved between the FIV_{Pet} and FIV_{Bang} isolates. These responses may have been directed against Gag epitopes in particular, since the amino-acid sequence difference between the FIV_{Pet} and FIV_{Bang} *gag* coding region (2.4%) is less than the difference observed at the *env* coding region (21%).

Overall, these findings resemble those of ALVAC–HIV-1 trials in macaques [21]. Macaques immunized with ALVAC–HIV-1 recombinants and boosted with subunit proteins were partially protected or had a delay in infection upon challenge with distinctly heterologous HIV-2. Prior to challenge these macaques exhibited HIV-1 cell-mediated responses. However, cross-reactivity of CTL responses against HIV-2 was not evaluated. Furthermore, serum samples obtained prior to challenge contained high titres of HIV-specific antibodies including VNA that effectively neutralized HIV-1 but failed to neutralize HIV-2 *in vitro*. In our study, immunization schemes employing recombinant ALVAC-based FIV vaccines in combination with ICV boost induced immunity effective against a homologous FIV strain as well as a heterologous strain from a different subtype. Although the exact immune correlates of protection remain to be identified, the current results suggest that antibody responses together with cell-mediated responses may be responsible for the partial to full protection observed in this study. To this end, results from this study shed promising light onto the development of broad-range vaccines against FIV and HIV.

Acknowledgement

The authors thank Chiron, Inc., for the adjuvant gift.

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